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HANDBOOK OF MICROSCOPICAL TECHNIQUE

HANDBOOK OF MICROSCOPICAL TECHNIQUE

For Workers in Both Animal and Plant Tissues

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WITH FORTY-THREE ILLUSTRATIONS

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PREFACE

In two previous texts, General Cytology* and Special Cytology,† edited by Dr. Cowdry, experts in various fields of biology have presented the results of their investigations. In the present work, which extends the series, the *methods* involved in these and similar studies are given.

There are two general needs to be met by a work of this sort. The first is that of the inexperienced worker who requires specific directions which he may apply with confidence to the general run of material, with the expectation of getting desired results. (Part I of this book outlines such standard methods.)

The other requirement is that of the experienced investigator who seeks the latest approved methods for the accomplishment of special technical results. (Part II presents this type of material.) In order to meet such a need as this, involving, as it does, the intimate knowledge of varied and often involved processes, the method of cooperation employed in the above-mentioned books is utilized. Workers highly experienced in the use of specialized methods describe these in detail. Since such directions will be consulted under terms which apply to the particular method or material it seems best to arrange these in appropriate chapters so that they may be reached directly as a group. In a discussion of these special methods, by a system of cross references, given in the text, to the first part of the work, unnecessary repetitions of the descriptions applying to general processes are often avoided. Also through the cross references between these special articles it is possible to follow out completely the methods which can be applied to any particular type of material.

The individual chapters and sections have been left largely as they were sent in by their authors, and this has resulted in some variations of statement extending sometimes even to open contradictions. So long as our procedures have only an empirical basis, judgments will continue to vary.

If one might draw a moral after reviewing, in an editorial capacity, the stated details of microscopical technology, it would be to the effect that while responsible for tremendous advances in our knowledge of living things, it is sadly in need of exact scientific bases for its own dicta. For precision and definiteness of results, microtechnique is now, and probably always will be, largely dependent upon individual skill, patience and judgment, but until we know just why a fixative or a stain produces a given result under one set of conditions and a different one under others, our interpretations lack an essential element of authority. There is much to be hoped from the present interest of chemists and physicists in biological

* General Cytology. Ed. by E. V. Cowdry, Chicago, 1924.

† Special Cytology. Ed. by E. V. Cowdry, N. Y., 1928, 2 Vols.

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matters, if they will submit their conclusions to the review of those intimately familiar with the conditions under which organisms live and operate.

Because this is a handbook for practical use there is no historical treatment and generally only such a bibliography as is necessary to complete an understanding of indicated methods. It is assumed that the book will be of interest particularly to workers in bacteriology, botany, cytology, embryology, histology and pathology, but the presentation of general methods in Part I makes it useful to any student of microscopic anatomy.

Limitations of space make it impossible to describe the instruments and apparatus required for microscopical studies, but those interested will find in the work by Simon H. Gage* full information on the subject. For the same reason there are few indications given regarding the source of biological materials, but in Guyer's "Animal Micrology"† there are numerous good directions of this character. In the encyclopaedic work edited by R. Krause‡ there are exhaustive discussions of many topics briefly treated in the present work. The Vade-Mecum of Lee§ is a standard reference. For a detailed review of the chemical basis underlying microtechnical processes the work by Gustav Mann‖ is very helpful.

It is a pleasure to acknowledge here the cordial cooperation of all the contributors, which has much lightened editorial drudgery. To Dr. E. V. Cowdry I am much indebted for consulting with the publishers, during my absence from the country, while the book was in press. For much expert bibliographic and secretarial assistance I have to thank my assistant, Miss H. Irene Corey. For reading the proof of the entire book I am under obligations to my wife, to my daughter, Mrs. Ruth M. Thompson, and to Miss Corey. Finally it is a pleasure to acknowledge the cordial and sympathetic cooperation of Mr. Hoeber and his assistants.

C. E. M.

PHILADELPHIA, PA.
October, 1928.

* Gage, S. H. The Microscope. Dark field ed. Ithaca, N. Y., 1925.
† Guyer, M. F. Animal Micrology. Ed. 2, Chicago, 1917.
‡ Krause, R. Enzyklopädie der mikroskopischen Technik. Ed. 3, Berlin, 1927.
§ Lee, A. B. The Microtomists' Vade-Mecum. Ed. 9, London, 1928.
‖ Mann, G. Physiological Histology. Oxford, 1902.

INTRODUCTION

Judged by the quality of the preparations used as a basis for many investigations, the importance of technical processes is not fully appreciated. The value of the results obtained from any microscopical study is directly dependent upon the quality of the technique employed in preparing the material. For this reason it is of utmost importance that every precaution be taken to secure the most accurate preservation attainable of normal conditions. It is unfortunately true, also, that no matter how highly developed a technique may be, faults in its application may entirely invalidate the results. It is not possible, therefore, by any mere statement of a process, to insure a high quality in the results of its use since so much is dependent upon the judgment and experience of the person employing the method.

However, it is feasible, by a clear statement of the steps involved, and of the results obtained by others, to indicate with some definiteness the course of procedure which should be followed. If the beginner could submit the results of his operations to one experienced with the method he would get definite suggestions of the highest value. In most cases the older worker, if convinced that the novice is really in earnest in his efforts, would give him sufficient of his time and experience to set him on the right way.

In any method the steps concerned are highly interdependent and failure at any one point is very apt to make ineffective the care exercised in the operation of other parts of the process. Accordingly there is no factor involved which is so insignificant that it may be overlooked.

It is fortunately the case that, after long experience, there has been developed a more or less standard technique which can be applied in most instances. The advantage of having such a common method to resort to lies in the fact that it affords a basis of comparison between different materials prepared in the same manner. In many cases this is of the greatest value because variations in technical methods often lead to widely different results.

However, it is quite impossible to apply any method effectively without understanding the reason for each of its steps. Mere application of a method by rote, with the expectation that thereby an accurate or standard result will be achieved, is quite a wrong procedure. There are so many factors involved in the use of reagents with protoplasmic material that an appreciation of the relations set up in each case is highly desirable. Often very serious inconveniences can be avoided by a clear understanding of just what is essential in the operation. To illustrate this point a very simple case may be instanced. Many beginners have great difficulty in making

paraffin sections adhere to the glass slip.* Much valuable time is wasted in trying out various expedients when an understanding of the essential requirement of the process would immediately lead to proper practices.

There are only two elements concerned in this step which require consideration. In the first place, the glass has to be chemically clean, which may be easily tested by noting whether or not the dilute albumen water adheres as an even film over the entire surface. If this be accomplished the next consideration is to see that the paraffin ribbon is completely extended so that at all points it ultimately comes down into intimate contact with the glass surface. This can also be tested after the complete evaporation of the water by examining the section on the reverse side. If the ribbon has been completely spread each section is seen in its entirety applied closely to the surface of the glass. If it is not in contact with the glass an air space is clearly observable. Thus, by applying two simple tests, one may be assured before removing the paraffin that the sections are going to adhere.

Similarly, in every other step of the process, such definite requirements must be met and for most of these there are clear tests. An understanding of the factors involved in each case accordingly makes one certain of results.

For the beginner the one sure method of progress lies in a careful study of the errors that develop and their elimination through an understanding of the principles which have been violated. In the beginning, therefore, progress is merely the perpetration of a series of errors and their correction as indicated. In order that this desirable end may be reached it is best to keep very careful records of all processes, noting the time element, the concentration of reagents, the interrelations of the steps, the nature of external conditions, and the appearance of the material as it goes through the process. Cards on which these records may easily be made have been designed,† and it is suggested that the beginner either make use of these or that he prepare others suited to his own particular requirements. At any rate, the necessity for accurate and complete records cannot be escaped.

The conditions to be met in the preparation of microscopical slides are set by the nature of the material to be studied and by the limitations of the instruments used for their study. In most cases the material is a mass of unstable colloidal substance, protoplasm, prone to rapid disintegration and of a size and character unfavorable for direct observation. It is generally necessary that this (1) be so treated as to undergo no essential structural

* Confusion and uncertainty arise from the use of one term for two different objects and it is an unjustifiable practice. Accordingly, contrary to general practice, the glass upon which the sections are spread, usually 1 × 3 inches in dimensions, will be called the "slip"; the term "slide" being restricted to the mounted preparation, consisting usually of the object surrounded by an appropriate medium and lying between the "slip" and "cover glass."

† Scammon, R. E. *Kan. Univ. Sci. Bull.*, 1908, iv, 4.
Hance, R. T. *Trans. Am. Micr. Soc.*, 1916, xxxv, 1.

change, but at the same time be so physically altered as to lend itself to manipulation; (2) that it be reduced to dimensions favorable to microscopical observation; (3) that the diverse elements present be colored differentially so as to become more strikingly apparent; (4) that the portions thus treated be mounted between pieces of glass of a convenient size and within a medium which will preserve them and present correct conditions of light refractions. These four requirements give rise to a series of processes, each of which is characterized by its own peculiar manipulations. They are described in their general application in Part I, and in their special uses in the succeeding chapters.

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PART I

GENERAL METHODS

CHAPTER I

SECTION AND NON-SECTION METHODS OF PREPARING MICROSCOPICAL SLIDES

C. E. McClung

Methods of Reducing Material to a Physical State Adapted to Microscopical Observation 3. Section Methods 3. Non-section 5. Detailed Description of Methods 6. Paraffin 6. Collodion 27. Freezing 29. Smear 30. Stretching 32. Teasing 33. Grinding 34. Macerating 34.

A. METHODS OF REDUCING MATERIAL TO A PHYSICAL STATE ADAPTED TO MICROSCOPICAL OBSERVATION

It is only rarely that material is naturally in a condition to be examined under the microscope without some manipulation. In general it is necessary to so reduce the mass as to permit light to pass through the object, since this is the manner in which most microscopical observation is conducted. There are two general means by which this requirement may be satisfied; first, the section method, which is the cutting of the mass of material into thin sections usually by means of a knife, so that light may pass through; and, second, non-section methods, whereby the mass of material is separated by some physical or chemical means into sufficiently small particles so that light may pass through and around them. Of these, the method of sectioning is the one most commonly employed.

I. Section Methods

The modern methods of cutting sections represent a gradual development from the earlier simple ones up to the present highly developed and more complicated procedures in common use. The older ones still find limited application in some cases.

1. Unsupported Material. Naturally the first means employed was simply cutting sufficiently thin slices from the fresh material by means of a razor. This is now restricted largely to hasty examinations of plant tissues. (For a description of the method see p. 123).

2. Imbedded Material. It was soon found necessary to support the mass of tissue to be cut in some way, which was done by some form of imbedding. At first a support, such as would be afforded by pith or cork, was employed, but later the mass of material was included in melted paraffin or wax. These procedures, however, merely supply a support for the external surfaces of the tissue and in no wise contribute to the rigidity of the interior of the mass. They therefore are rarely used.

3. Infiltrated Material. Methods of infiltration were then developed. In these the specimen is so treated that the supporting material may enter

intimately into the mass and give it support at every point. Only by the use of such means has it become possible to secure serial sections of the uniform thickness required by modern microscopical methods of study. There are two media which are commonly employed for infiltrating, paraffin and collodion.* These have each their advantages and disadvantages and the method chosen must have regard to the character of the material and the purpose for which it is to be used. The details of application of these two substances for infiltration will be found under appropriate headings, but it may be desirable to indicate here the chief characteristics of the two.

Paraffin as a Medium. The advantages of paraffin are that it readily permits one to secure thin sections preserved in a fixed serial order. The disadvantages are that large paraffin sections cannot readily be obtained and that some shrinkage occurs from the action of the clearing and infiltrating media, especially at the temperature required for keeping paraffin melted.

Collodion as a Medium. The advantages of the collodion method are that large sections may readily be cut and in the absence of heat at any stage of infiltration shrinkage is avoided. Collodion may also be indicated for certain tissues which become hard when paraffin is employed for infiltration. It is also true that, following certain fixatives, collodion may preserve the finest cytological details in cells which by paraffin infiltration become seriously altered. It is therefore desirable in every case to try both the paraffin and collodion methods if the very best results are desired. The disadvantages of collodion are that thin sections cannot readily be cut and the process of sectioning is relatively slow and in some ways more difficult, especially if serial sections are desired.

Other Media. For special purposes gelatin, soap and other media may be better adapted than the two commonly used. (For an account of some such cases, see p. 125).

4. Choice of Medium. As is indicated, the choice of the infiltrating medium must depend on the nature of the material and the purpose for which the sections are cut. By considering the advantages and disadvantages of the various media it should be possible to select one which will be most suitable.

5. Grinding. This method is applicable only to such dense substances as bone or teeth and therefore has a very limited use.

6. Characteristics of Section Methods. The advantages of sections as a means of microscopical analysis of structure are that the elements are preserved in their normal relations to each other and that they are in so thin a layer that the intimate details of cell and tissue structure may be observed. The disadvantages lie in the fact that in thus reducing the mass to thin sections, elements are cut through regardless of their natural boundaries and fragments of cells are then presented for study at certain points.

* Collodion, celloidin, parlodion, etc.

No absolute rule can be given with regard to the proper thickness to which sections should be cut to secure best results. It all depends on the purpose in view and on the character of the material. In general, it may be said that, for best staining results, the cells should be cut through, that is the sections should not exceed the average diameter of the cell, but it is also the general rule that for the study of very fine details of structure, thinner sections are best. The reason for this is that differentiation of stains, especially the iron hematoxylin stain, can be best secured in the absence of any great variation in the character of the material in the section. Thin sections reduce this variation. On the other hand when it is desired to study general relations, thick as well as thin sections are desirable. In chromosome studies, where smear preparations are not convenient, thick sections will preserve the entire complex in certain cells so that there is no danger of counting fragments. Very thin sections range from 1 to 5 μ, medium sections from 5 to 10 μ, and thick sections from 10 μ up to 20 or 30 μ. Sections of average thickness will be found generally most convenient for study.

II. Non-section Methods

If material is prepared by non-section methods the choice of means is strictly limited by the physical characteristics of the tissue. There is, therefore, less flexibility here than in the case of section methods which can be applied to almost any type of tissue.

1. **Smearing.** Of the non-section methods, the one most commonly employed is known as the smear method. The use of this is restricted to tissues of a fluid or semi-fluid character which may be spread in a thin film upon a glass surface. Such tissues as blood lend themselves most readily to this operation and in this particular case almost require the application of such a method.

2. **Stretching.** This method is rigidly limited in its application, being useful in the case of natural membranes which may be extended in so thin a layer that they are observable like sections. The only requirement here is a means for holding the membrane stretched while it is fixed. After that it is treated like a section.

3. **Teasing.** The operation of teasing can be applied only to fibrous tissues such as tendon and muscle. By means of needles the connective tissue is combed away from the fibers leaving them individually free or collected into small groups.

4. **Macerating.** The three previously mentioned methods are al physical in character while maceration is a chemical method of dissociating elements. In its use a weak fixative is employed which differentially affects the various tissue so that the connective tissue becomes soluble and leaves free the elements which it bound together. This method may be used with a wide variety of tissues.

5. **Choice of a Non-section Method.** As has been stated, the non-section methods are limited in their use by the physical characteristics of the tissue and so the selection of any one of them is definitely indicated and does not require further discussion.

6. **Characteristics of Non-section Methods.** As contrasted with section methods, the non-section methods, aside from stretching, present individual elements entire, but dissociated from their normal relations. It is obvious, therefore, that when possible it is desirable to use both types of procedure.

B. DETAILED DESCRIPTION OF METHODS

I. Paraffin Method

Since this is the method which is most commonly employed, the complete series of processes involved will be stated in detail and will serve as a basis upon which to consider other less used methods. These steps follow in order, are mutually interdependent, and so have to be considered as a whole. For purposes of convenience, however, it is desirable to separate the entire account into its steps in order that these may be better presented.

While the general directions supply the information necessary for the accomplishment of certain results with a given method, there are so many details involved that a definite statement of the application of the paraffin method to a particular piece of tissue will be given. Because much of the technique is applied to visceral organs, one of these, the liver, will be used as an example.

1. **Killing** is the act of stopping the vital processes of an organism. It might seem to be a relatively unimportant detail but it turns out to be quite the contrary, for in some cases, at least, the entire appearance of the cell depends upon this step. For instance, in the case of the first spermatocytes of the Orthoptera, if the animal is killed by an anesthetic, the cytoplasm has a fine fibrous character in the metaphases and the chromosomes are well spread and clear in outline. On the contrary, if another animal is treated with cyanide, the cytoplasm at a corresponding stage is coarsely granular, the spindle is not over half the length of that in the other case, and the chromosomes are closely drawn together and indistinct in outline. Since these are elements of the greatest significance in the structure of the germ cells, it is quite apparent that the method of killing is of the utmost importance. In many cases this may not be so significant a step, but it is something which should always be considered. In general, the use of an anesthetic is recommended, particularly in the case of smaller animals. Larger animals may be killed by a blow on the head or by severing the spinal cord. In every instance the operation should be completed as rapidly as possible.

If, for example, a rat is used, the animal should be placed in a glass cylinder with a tight fitting cover. Drop into this a piece of absorbent cotton saturated with chloroform.

Allow the animal to remain until unconscious and then remove it from the vessel. Open the abdominal cavity by a median ventral incision if visceral organs are desired. From the organ cut a segment which should be about 5 mm. in linear dimensions. If it is desired to carry through any other pieces of tissue they should be removed at the same time.

2. Marking. This is a means of attaching a clear and unequivocal designating number or character to a specimen. As soon as the material to be processed is removed from the body of the animal it should be definitely marked in some way so that it may not be mistaken for any other specimen. In many instances it is important to preserve the animal from which the material has been removed, and in this case the same designating character should be placed on the animal and upon the part removed from it. In case any considerable number of specimens are being investigated, the simplest means is to mark each with a serial number so that there may be no duplications. Such lot numbers are conveniently entered in a book, just as are the accessions in a library. Later, for purposes of convenience, classifications according to the subject matter can be made, but the existence of one unequivocal designation for each specimen in the collection is of the greatest importance.

It is sometimes difficult to carry through the designating mark with the material, but it may be done by employing a vessel for each specimen. A very convenient way when many specimens are to be prepared at the same time, is to put the lot number on a convenient sized piece of paper with a pencil or water-proof India ink, and on the reverse side of the paper the fresh material just removed from the organism. Then the two are immediately immersed in the fixing fluid. In this way the piece of paper is firmly attached to the specimen by coagulation and may then be carried through the subsequent processes without danger of dislocation. If material, such as that from plants, will not adhere, it may be made to do so by using a little Mayer's albumen. If record cards are employed, the lot number is placed upon the proper card and, as the material passes through the subsequent steps, these are carefully recorded in order. Danger of neglecting to keep records of essential steps is thus avoided. The card, when completed, forms a part of the permanent record relating to the specimen and may be consulted at any time.

3. Fixing is the process of preserving, by means of coagulation, the normal structural characters of organs, tissues and cells. Before fixation the greatest care must be exercised in manipulating tissues. They are easily injured by pressure and in many cases it is best to fix in situ. Where this is not possible the operation of removal should be conducted with the greatest care and the tissue disturbed as little as possible. In general, the smallest piece of tissue that will suffice should be used because the fixative penetrates larger pieces with difficulty. As noted under different fixatives, the degree of penetration varies. Picro-formol-acetic combinations penetrate readily, as does the acetic acid, alcohol and chloroform combination,

but osmic mixtures have very slight power of penetration. On the average, pieces of tissue from 2 mm. to 8 mm. in diameter are best for all purposes.

The process of fixation is one of the most important in the series. Upon the character of its operation all the remaining steps and the ultimate result depend. The endeavor here is to preserve in a permanent form, as nearly as may be, the exact configuration of cells and tissues in the living state. Obviously this cannot be exactly accomplished because living protoplasm is a gelatinous or semi-fluid material, and after fixation it becomes a solid. Since, however, this change is accomplished by the process of coagulation it is possible to preserve in the solid form essentially the same relations that obtained in the living state. It is only after considerable experience that one may accurately judge the operation of fixation, but in general it may be said that any evidence of shrinkage or swelling indicates that the process has not been perfect in its operation.

It is claimed by some that, by the very nature of fixation, the normal structure has been destroyed, but a careful comparison between living cells and similar cells after fixation has demonstrated that the picture of the preserved material accurately represents that existing in life. This is a matter of fundamental importance, for, unless we are studying normal relations in the preserved material, nothing is to be gained by its use. It may be said, however, with every assurance, that a properly prepared specimen affords an accurate means for determining normal structural conditions. Of course it is necessary to interpret microscopical images and, unfortunately, by speaking in terms of solid substances in cells, a misinterpretation is involved. It is customary to refer to certain aggregates in cells as "granules" when, as a matter of fact, in the living cell these are droplets of varying degrees of density. If it is understood what is meant by such terms, however, there is no danger of misconception.

To be effective fixation must be accomplished as rapidly as possible, before any post mortem changes have taken place in the tissues. Therefore it is often desirable to apply the fixing fluid directly to the part in the body of the animal. This can easily be done in small specimens by opening the body, and in large ones by means of injections. There are very few instances in which a delay in applying the fixative is desirable. The general rule is to fix immediately and avoid any change due either to temperature or evaporation.

Reagents. The reagents chosen should be adapted to the purpose for which the material is used. Some fixatives preserve nuclear conditions especially well, others those of the cytoplasm. In this general description of processes it will be assumed that the nuclear conditions are primarily in mind. The particular fixative that may best be used, again, depends upon the nature of the material. In some cases this requires a reagent which penetrates with extreme vigor and rapidity. In others it is necessary to

choose one which is slower and more delicate in its operation. What is required in each case can be determined only by experience. In the following description it will be assumed that the material considered is of the character found in some visceral organ in which extreme difficulties in penetration will not be met. Under these circumstances extended recent experience has indicated that one of the picro-formol-acetic mixtures may be selected with assurance of getting at least a good fixation in almost every instance. In most cases it is the best that can be obtained.

The three substances, picric acid, acetic acid, and formalin have individually different effects upon protoplasm. Thus picric acid alone shrinks it while both acetic acid and formalin have a swelling action. The proportions of these reagents must therefore be adapted to the particular kind of material treated. Only previous experience, or a general knowledge of the nature of the material, will make it possible to choose at first the particular combination which will be most effective. Commonly, however, the fixation obtained with Bouin's original formula will be good (p. 424). In case very resistant tissues are involved, the two fluids invented by Carnoy (p. 422) will commonly be most effective.

In recent years it has been found that certain non-coagulating substances like urea and sugar, when added to fixing fluids, have specific results. The choice here again depends upon the effect desired, which can be learned only by experiment. However, in general it appears that the addition of something like 2 per cent of urea to certain fixing fluids materially improves the character of their operation. In the case of plant cells various sugars show specific effects (p. 148).

Assuming that the specimen has been appropriately excised and marked and that the proper fixing fluid has been prepared, the next step is to apply the fixative under appropriate physical conditions and for the proper length of time. Ordinarily the fixative may be used at room temperature, but for certain definite results, higher or lower temperatures may be necessary. It has been found, for instance, that adding urea to Flemming's fluid and fixing Orthopteran cells at a temperature of 0°C. reverses the relative density of nucleus and cytoplasm as compared with the results of ordinary fixation—that is, the nucleus appears as a very dense body. For further discussion of this topic see Flemming's fluid and Allen's fluid (p. 424). The length of time required for the best fixation depends, among other things, upon the nature of the material, the character of the fixative and the temperature. Some fixatives like the osmic acid mixtures, over-fix if employed for too long a period, while others, like certain picro-formol-acetic combinations, may be allowed to act indefinitely without injury. As a general guide it may be said that for ordinary tissues in pieces not over 4 mm. in diameter, fixation will be accomplished in from two to twenty-four hours. In view of the flexibility of the P.F.A. mixtures it is desirable in the beginning to employ them because the length of their operation has

very little influence on the character of the end result. That is one of their many advantages.

Since the reagents involved in these fixations are vigorous chemicals, it is generally necessary to use glass or porcelain containers and to avoid bringing any metal instruments into the fluids. If the specimen has been fastened to a piece of paper with the designating number upon it, it is convenient to float the material on the surface of the fixing fluid where it will be held by surface tension. This is an advantageous position for the material because it always remains at a normal concentration of the fixative, whereas if it lies at the bottom of the vessel in a limited amount of fluid, the water extracted from the material dilutes the fixative as time goes on. To avoid any such contingency as this it is well to use a sufficient quantity of the fixative so that additional water will not affect its concentration. At the end of the process of fixation the material is completely saturated with the reagent and a certain proportion has chemically combined with the protoplasm.

Hardening. The stronger fixatives, like the chromic acid combinations, mercuric chloride mixtures, etc., harden sufficiently so that no subsequent treatment is required. In the case of some of the more delicate fixatives, however, further treatment with alcohol of high concentration or with bichromate of potash is required. The material should be kept in 95 per cent alcohol or in 5 per cent bichromate until the desired consistency is reached, a period varying considerably with the size and character of the specimen. There is always a danger of excessive hardening when treating unfamiliar material, but this can be avoided by testing at intervals. Due consideration should be given to the question of whether sectioning is to be done in paraffin or collodion.

4. **Washing** is the process of removing the excess fixative from the specimen. The medium to be employed here depends upon the nature of the fixative, but commonly it is water following aqueous fixatives. Only in the case of fixations with such weak agents as picric acid in the absence of formalin, is it necessary to wash out with alcohol in order to avoid maceration. Washing is completed when approximately all of the uncombined fixative is removed from the tissue. It is sometimes difficult to determine when this has been accomplished, but with the picro-formol-acetic mixtures it can be assured when the yellow color of the picric acid is no longer strongly manifest in the washing fluid. As a general rule, for fixatives like Flemming, Hermann (p. 423), etc., it is desirable to wash out at least as long as the time involved in fixation and to be on the safe side it should in most cases be longer than this period, if not to exceed twenty-four hours. It is possible to facilitate the removal of the fixative in some cases by adding certain substances to the washing fluid. Thus with picric acid, the presence of a small quantity of lithium carbonate lessens the washing period, while with mercuric chloride, iodine similarly hastens the process. It must be remem-

bered also that in subsequent steps a continuation of the process of extraction may be counted upon. In most cases, moreover, a small quantity of the fixative will do no damage up to the time of staining. This is especially true of the P.F.A. mixtures. On the other hand, when mercuric chloride has been used, complete removal is necessary, otherwise the crystals remaining produce artifacts and also injure the cutting edge of the microtome knife. If washing has been incomplete, in cases where chromic acid has formed a part of the mixture, the staining reaction with hematoxylin may be reversed in the nucleus. The general rule, accordingly, which should be adhered to, is to remove as completely as possible all traces of the fixing agents.

To carry out the process of washing, in the example selected, the tissue is removed to a tall Stender dish filled with water, where it may again be floated on the surface. Only the excess of the fixing fluid is removed in the water, which may be done in about an hour. After this it is transferred to vessels containing successively 30 per cent, 50 per cent and 70 per cent alcohol. The intervals in these various strengths of alcohol may be, for the first two, an hour each, and for the 70 per cent a sufficient length of time to remove most of the fixative. A test for this is furnished by the extraction of the yellow picric acid. This may be hastened by adding 10 drops of saturated aqueous solution of lithium carbonate to each 30 c.c. of 70 per cent alcohol. It is sometimes desirable to remove the fixative completely but under present circumstances a small quantity remaining in the tissue will not be disadvantageous.

5. Staining in Toto. At this point it is sometimes advantageous to stain the mass of tissue entire, or, as it is commonly designated, in toto. Not all staining agents are useful for this purpose. Amongst the best are the carmine and cochineal mixtures. Hematoxylin combinations are less effective and rarely can aniline stains be employed at all. One of the best stains for this purpose is the alum-cochineal mixture, especially for embryos (p. 468). Obviously, in this process, it would be necessary to employ a reagent which does not over-stain. Where material can be treated in toto, it is, of course, a very great advantage because the sections are all ready for mounting as soon as the cutting and attaching to the slip are accomplished. For embryological purposes this is a very good and frequently used method, but in cytological work it finds only limited application.

To stain the piece of liver, place it, after dehydration, in alum-cochineal, diluted one-half, for five to ten hours. This gives a nuclear stain and if the picric acid has not been completely washed out a double stain will be obtained in which the yellow of the picric acid contrasts fairly well with the red of the cochineal. After staining, wash the specimen for thirty minutes in water to remove the contained alum which otherwise would crystallize in the alcohols and cause difficulty in sectioning.

6. Dehydration is the process of replacing water in the specimen with alcohol. In most cases the material, up to this point, has been in aqueous solutions, but it now becomes necessary to remove the water. This is usually accomplished with alcohol. It is not a matter of indifference

how this is done, for the attraction between water and alcohol is so violent that, in mixing, diffusion currents often severely damage the tissues. For this reason it is customary to proceed gradually in the removal of water in either one of two ways: (1) by passing the material through ascending grades of alcohol of 30 per cent, 50 per cent, 70 per cent, 83 per cent and 95 per cent, and sometimes up into absolute alcohol; (2) increasing the concentration of the alcohol gradually by adding it slowly in small quantities to the water in which the specimen is placed. Whatever method is employed for adding the alcohols, arrangements should be made to secure a mixing of the fluids, since the lighter alcohol remains on the surface. A convenient way of doing this is by means of capillary syphons which add the alcohol to the water slowly, a drop at a time. In this case, facilitate mixing by agitation, either by stirring devices or by the passage of a stream of air bubbles through the mixture. A vessel charged with compressed air may be connected with a small glass nozzle which reaches to the bottom of the fluid, and by regulating the flow of air the fluids may be kept completely mixed. It is advantageous to draw off portions of the mixture at intervals, thus increasing the concentration of the alcohol (p. 188).

Still another way is to suspend the specimen, in a small quantity of water, in a vessel with a membranous bottom in a tall cylinder of 95 per cent alcohol. The water, because of its greater specific gravity, descends to the bottom of the cylinder, leaving the specimen bathed in the stronger alcohol at the top. This is not so satisfactory because the interchange between the water and alcohol may be violent.

At the end of dehydration the water is completely removed from the specimen and in its place is found absolute alcohol, if this be used. It is very important to see that this end is reached and sufficient time should be given in the final strength to secure this result. There is little danger of shrinkage in 95 per cent alcohol, and for this reason it is a safe practice to permit the tissue to remain in it for twenty-four hours or longer.

The various grades of alcohol have their particular effects upon the material and some of these have practical advantages. In lower grades maceration may be accomplished, if this is desired, while in the intermediate, such as 70 per cent, material may be kept indefinitely. Beyond the strength of 70 per cent, especially in absolute alcohol, hardening occurs, accompanied by shrinkage, and for this reason it is not desirable to prolong action here any more than is necessary.

While it is possible to proceed directly from 95 per cent alcohol or absolute, into xylol, it will be found a matter of convenience to pass the material into a mixture of equal parts of 95 per cent alcohol and xylol. This mixture should be entirely clear and if it is not so it indicates that the alcohol has fallen below 95 per cent and sufficient absolute should be added to make a clear mixture. By using this intermediate step greater assurance is gained in respect to the completeness of dehydration.

7. Clearing is the process of removing the alcohol in the specimen with some fluid miscible with paraffin. In order to carry the material from alcohol into paraffin it is necessary to interpose this intermediate step. For this purpose some substance must be used which, on the one hand, mixes readily with alcohol and, on the other, dissolves paraffin—commonly one of the hydrocarbons, such as benzol, xylol or toluol. Chloroform, turpentine or any of the essential oils can be utilized, and for many purposes aniline oil is advantageous. If the essential oils are used the piece of tissue becomes entirely translucent when the alcohol is completely removed, hence the name of the process. That is true also of aniline oil, but xylol or chloroform do not produce so marked a clearing effect and it is therefore more difficult to determine when their action is complete.

Because it is sometimes difficult to secure full dehydration within a limited time, the practice of clearing in aniline oil has been growing. If aniline is employed the tissue may be transferred to it from 95 per cent alcohol or even from a lower grade (p. 190). To avoid carrying the aniline over into the paraffin, treatment with chloroform is given the tissue until most of the aniline is removed. Two changes of five minutes each is sufficient.

In clearing reduce the time of the operation to the shortest period since shrinkage occurs. In most cases, change the clearing fluid at least once in order to avoid carrying over any alcohol into the paraffin. At the end of this process the alcohol is completely removed and all portions of the tissue are filled with the clearing agent. A piece of tissue, such as is being described, would probably require two or three hours for complete removal of the alcohol.

8. Infiltrating is the process of replacing the clearing agent in the specimen with melted paraffin. Paraffin at ordinary temperatures is a solid and in order that it may penetrate the tissues and replace the clearing agent it is, of course, necessary to make it a fluid by means of heat. This has introduced a complication of considerable magnitude, which, however, may be avoided by a very simple means. It was formerly customary to employ very elaborate water baths with thermo-regulators in order to keep the melted paraffin at a constant temperature. The same end may be readily accomplished in a much simpler and more effective fashion by placing the paraffin in a cylindrical vessel, like a tumbler, over which is suspended an incandescent lamp which will give sufficient heat to melt the upper portion of the paraffin. The 150 watt nitrogen filled bulbs on the market now do this very well. In practice, the tumbler, three-fourths full of paraffin, is placed beneath the incandescent bulb which is so adjusted in relation to it that a layer approximately an inch in depth remains in a fluid condition. Into this is transferred the specimen saturated with clearing fluid.

Under the conditions of the operation just described the piece of tissue drops to the bottom of the melted paraffin and comes to rest in a layer just at the melting point. If the temperature increases the paraffin melts down further, but the specimen drops with it and so it never can become overheated. At the same time the clearing fluid, being lighter than the paraffin, rises to the surface, where the temperature is greatest and is thus more rapidly evaporated. The piece of tissue remains in the melted paraffin in general somewhat longer than the time required to clear the specimen. There is no way of telling when the clearing fluid is completely removed by the appearance of the material, and so some such arbitrary rule has to be followed. While immersed in melted paraffin the material constantly undergoes shrinkage. The time of its operation is therefore reduced to a minimum.

There are many grades of paraffin determined by the temperatures at which they melt, and the choice of the particular one to be employed depends upon the character of the material and also upon the temperature at which the sections are to be cut. The general principle involved here is to select a grade of paraffin which, at room temperature, somewhat nearly approximates in density the specimen itself. If there is a wide difference in this respect the sections will be imperfect through compression, if the paraffin be too soft; or through cracking and breaking if it be too hard. Sections in soft paraffin form ribbons well, but are difficult to handle. Very hard paraffin may fail to ribbon except at high room temperature. A marked difference in density between object and paraffin results in the generation of troublesome static electricity in sectioning. Under ordinary conditions a grade of paraffin melting between 54°c. and 60°c. is most useful, but in case very hard material is to be cut, a paraffin of higher melting point is necessary. Here enters another complication, however, because if a hard paraffin is cut at a temperature widely removed from its melting point it becomes brittle and does not section well. Under these circumstances it is necessary to raise the temperature of the room in which the cutting occurs in order to bring about a proper relation between room temperature and density of the paraffin (p. 476). For ordinary room temperatures the grade of paraffin chosen for specimens, such as a piece of liver tissue, should be of a melting point of about 58°c. The specimen would probably be completely infiltrated in three hours. If many specimens are being carried through at one time, transfer them into a fresh bath of paraffin. To completely evaporate the dissolved clearing agent allow the paraffin to remain in the melted condition for some time after the specimens are removed. Occasionally the jar of paraffin should be melted completely so that any suspended particles may sink to the bottom. No attention to the tissue is required while in the paraffin and other work may be carried on advantageously during this interval. By a careful arrangement of the different operations much time may be saved in this way.

At the end of the operation of infiltration the clearing fluid has been completely removed and all interstices of the specimen are filled with paraffin in the melted condition.

9. Imbedding is the process of enclosing the specimen in a convenient block of solid paraffin. The size of the block is determined in a variety of ways, such as forming paper boxes of appropriate dimensions or by manipulating metal blocks upon a glass plate so as to produce a chamber of proper size. Perhaps the best imbedding method of all is the one called the watch glass method. In this an ordinary rounded watch glass is very lightly coated with glycerin by rubbing a moistened finger over the concave surface. Into the glass, by means of a warm pipette, is placed a quantity of melted paraffin from the dish in which the specimen has been infiltrated. With proper manipulation a rounded body of paraffin may be built up by cooling the outer surface and slowly adding at the center additional amounts of melted paraffin. At the same time a thin film of hardened paraffin forms at the bottom of the dish and when the conditions are right the specimen is removed from the infiltration vessel by means of warmed forceps and deposited in the proper position within the mass of melted paraffin in the watch glass. Here it is oriented in relation to the table surface so that the plane of sectioning is thus indicated. Care should be taken to see that the amount of paraffin above the specimen is of some extent because on cooling there is considerable shrinkage, and the specimen should lie completely enclosed within the hardened mass of paraffin. By conducting these manipulations within the area heated by the incandescent bulb, convenient conditions of temperature may be found for good imbedding.

As soon as the tissue is thus arranged, by blowing on the surface of the paraffin a film can be produced and when this is of sufficient thickness so that the water will not easily break it, the watch glass with its contents is lowered slowly into a dish of cold water. When it is completely submerged the glass is allowed to drop to the bottom of the container. The purpose of this rapid cooling is to harden the paraffin before it has time to crystallize.

One advantage of the watch glass method over others is that in this process of cooling the mass of paraffin shrinks towards the center and is brought into most intimate contact with the material. If, on the contrary, the paraffin may adhere to the rigid walls of a vessel on cooling, it tends to draw away from the specimen and thus produces a difference in density in the immediate vicinity of the material.

After a time the glycerin film between the paraffin and the glass dissolves and the block of paraffin rises to the surface. If the specimen has been carried through upon a slip of paper with a lot number upon it, this can be read through the thinner layer of paraffin at the bottom and the material easily identified. Specimens thus imbedded may be kept indefinitely without change and cut at any time.

10. **Blocking and Trimming** are processes concerned with getting the specimen into a determinate block of paraffin which is of such a character as to produce readily a continuous series of sections when cut on the microtome. In preparing the material for attaching to the microtome, mount the block of paraffin with the contained specimen on some support which fits into the object holder. Most microtomes have metal discs that are designed for this purpose, but it is more convenient in general to use a cylindrical wooden block upon the end of which the paraffin mass is attached. A very convenient way to make this connection is to stand the wooden cylinder on end, after having first filled its pores with melted paraffin. A section lifter is warmed in a flame and laid upon the paraffined end of the wooden block and at once the paraffin mass is brought down upon this heated section lifter, which is then immediately withdrawn, thus bringing the paraffin and wooden blocks into contact. In this way the melted paraffin attaches the specimen firmly to the wooden support. Since the specimen was so adjusted that the plane of sectioning corresponds to the bottom of the mass of paraffin, the plane of cutting is now indicated by the upper surface of the wood. To bring about a firmer union between the paraffin and the wooden support, immediately after withdrawing the heated section lifter, plunge the mount into a vessel of cold water.

As a further preparation for sectioning, the excess paraffin about the specimen must now be removed. The manner of accomplishing this is as follows:

By means of a safety razor blade or similar cutting instrument, the paraffin is gradually trimmed up towards the specimen until there is produced a plane figure of four sides, two of which are parallel, the third at right angles to these two, and the fourth at a slightly acute angle with one of the parallel sides; in other words, a trapezoid with the specimen lying near the center. It is necessary to have two parallel sides, which on sectioning are the ones in contact with the cutting edge of the knife. The purpose of the inclined edge is to indicate one side of the ribbon by means of a notched contour. This is very helpful later in handling the segments of the ribbon.

In trimming the block, two points should be borne in mind aside from those already mentioned. First, do not extend the cutting below the level of the bottom of the material very far. The reason for this is that it is best to have as much support as can be gained, and, by removing no more paraffin than is necessary to clear the material, this is aided. Sometimes, when a very long piece of substance is being cut, it is desirable to trim only part way down its length and then by successive trimmings to carry the shaping to the bottom of the piece. The other point to be kept in mind is to trim the paraffin as near to the contained material as possible without at any time touching it. By having only a slight amount of paraffin around the substance its sections are brought very near together on the slide and thus a more compact mount is secured. If, however, the trimming exposes the material at any point the sectioning is more difficult and of course an injury is done to the specimen.

It is very difficult to orient and trim the block about a specimen which is of about the same color as the paraffin in which it is enclosed. It is therefore helpful, in case of a fixation which does not color the tissue, to

tint it in its passage through the higher grades of alcohol with eosin or some other bright color which will serve to mark outlines clearly. If the color is undesirable later it can easily be removed by leaving the sections sufficiently long in alcohol.

11. **Cutting** is the process of removing successive sections of uniform thickness, usually in the form of a ribbon, by the operation of a knife in a machine called a microtome. Not very much that will be helpful in learning proper methods of sectioning can be given by means of verbal description because so much of the facility necessary in this operation comes only through experience. However, there are some general features which may be mentioned and which will be of service. First, the specimen should be so mounted in the holder that the two parallel sides of the paraffin block are strictly parallel with the cutting edge of the knife at the moment of passing it. Next, the holder should be so adjusted that the plane of up and down movement corresponds to the plane through which the section is desired. The knife should be inclined at such an angle to the plane of sectioning that the paraffin strikes it only at the extreme edge. Most knives have a secondary plane of inclination extending only a short distance from the cutting edge and this is the one to be considered in arranging the inclination. On the other hand, the slope of the knife should not be excessive. It should be as nearly vertical as it is possible to get it while avoiding contact anywhere except at the cutting edge. The reason for this adjustment is that the more nearly vertical the knife is the less resistance it offers in passing through the specimen and the more nearly perfect the section it cuts. On the other hand, if the knife is not sufficiently inclined the block drags over the back knife surface below the cutting edge and injures the specimen. Too great an inclination of the knife edge breaks the sections and crumbles them. It is thus obvious that the proper inclination of the knife is an important matter. In order to present the most rigid cutting edge, the knife should be clamped in the holder as near to the specimen as is convenient. The better forms of microtomes have holders which are adjustable in this way. Make all of the adjustments on the microtome to secure the greatest degree of rigidity. Any movement, either of the knife or of the specimen, aside from that provided purposely, is inimical to good sectioning.

It is hardly necessary to say that only with a perfect cutting edge on a knife can good sections be obtained. Any nicks or irregularities will produce glaring defects in the section.

In cutting sections there appear certain faults which permit of suggestions as to causes, and remedies which may be applied. One of the most common faults is a ribbon which, instead of coming off perfectly straight, with sections of even dimensions, is curved. This form is due to the fact that each section is a little narrower on one edge than the other, which may be due to trimming the block so that the two edges striking the knife are not exactly parallel. Thus each section instead of being a trapezoid, is wedge-

shaped. On other occasions the curved form of the ribbon may appear even when the two edges of the block are parallel. This effect may be occasioned by greater density at one side of the block than at the other, in which case the denser half is wider and so produces the same result as converging sides in the block. If the knife edge is not uniformly sharp throughout a difference in section width may thus be produced.

If the sections are much less in diameter than the face of the block from which they are cut, there is a difficulty either with the sharpness of the knife edge or its inclination to the plane of sectioning. In extreme cases the section will be forced into folds that cohere so firmly that it is impossible to straighten the section out completely. If the room temperature is too low, the sections may break across at intervals. This effect is exaggerated if the inclination of the knife is excessive. The nearer the diameter of the section produced is to that of the block from which it is cut, the more perfect is the sectioning. Any excessive disproportion here should lead to an investigation of the causes before the sectioning is continued.

Sometimes the ribbon will cut perfectly for a while and all at once a slit will appear in the sections. In most instances this is caused by the accumulation of material on the edge of the knife which produces a break in each section and so splits the ribbon. Without removing the ribbon from the knife edge this place should be cleaned off with an upward motion of a soft stick. *Always avoid bringing any metal instrument in contact with the knife edge.* If this procedure does not remedy the difficulty, the split is probably caused by a fault in the knife edge, in which event the knife should be slid laterally so as to produce the cut in another place. An air-bubble or other fault in the paraffin block may produce a split ribbon.

One of the most annoying circumstances occurring in paraffin sectioning is the production of static electricity in the ribbon, which causes it to fly violently towards other objects to which it adheres. This may be avoided by seeing that there is not too great disproportion in density between a specimen and the paraffin, since the electricity is in part produced by differential friction in regions of the block; in other words, the paraffin must be sufficiently hard (58°c.) and the infiltration complete.

Irregularities in the thickness of a section may be caused by lack of rigidity in the knife or in the block, by irregularities in the feed mechanism or by variations in the speed with which the sections are cut. In general it is best to cut at a moderate rate of speed which is kept uniform throughout the process, avoiding any stops if this is possible. Under favorable circumstances the entire ribbon may be cut without breaking and with very little variation in the thickness of the individual sections. Obviously it requires a very fine mechanism to produce several thousand sections of absolutely uniform thickness. Even with the very best machines it is impossible to avoid irregularities unless every condition is favorable. There should not, however, be any marked variation in successive sections. If

it should happen that one section is very much thicker than the preceding one it is probably due to an irregularity in the feed of the microtome so that the block adjustment moves forward irregularly, to loose adjustment of knife or specimen, or to too vertical a knife.

These difficulties are a few of the many that may occur in the cutting process and each of them has a definable cause which, when discovered, permits of a remedy. It should, therefore, be the endeavor of the operator to study the difficulty carefully and determine its cause before proceeding with the sectioning operation. When very careful work is done on serial sections it is important to have the ribbons as straight and uniform as it is possible to get them. Much time is lost in passing from one section to another, when cells or structures which should be in alignment are so displaced that they fall without the field of a high power objective. A little time devoted to improvements in the process of sectioning may save a very large amount of work subsequently.

Manipulation of the Paraffin Ribbon. As has been noted, the character of the ribbon depends in considerable measure upon the rate and regularity with which the sections are cut. There are also some small manipulations which greatly facilitate the obtaining of a ribbon of the first quality. If it is at all possible the ribbon should be cut without stoppage and wound upon a cylinder entire. This is often difficult of accomplishment and if it becomes necessary to separate the ribbon it should be done, not at the knife edge, but at some little distance from it, leaving a short segment of the ribbon attached. In this way when the cutting is resumed the ribbon will be maintained and the danger of loss from the first sections rolling up will be avoided. In laying the ribbon down a uniform order should be used habitually. The order followed in writing, e. g., beginning at upper left hand corner of container, etc., is convenient.

In mounting the sections on the glass, care should be taken to see that the polished surface of the ribbon goes next to the surface of the glass. Sections adhere better when mounted in this way. If the block has been trimmed with one inclined lateral margin the ribbon will show a serrate edge which will mark that side of the sections. Thus with the one surface and one edge determined there will be no danger of inverting the order of the sections or of turning the segment upside down. It is of the utmost importance in serial sections to maintain the exactness of seriation.

If a number of blocks are cut at one time it should be the practice to mark upon the container carrying the ribbon the exact serial number which belongs to it. If this is not done it is impossible to avoid confusion. During all the processes it should be the practice to keep attached to the sections their exact designation.

12. **Spreading** is the process of extending sections by heat until all effects of compression in sectioning are removed. If the operation of sectioning has been a success the entire block may appear in a continuous

ribbon of sections. In this event cut the series into segments of appropriate length for temporary storage in some convenient container having a clean, smooth paper bottom. For mounting, the ribbon is divided into segments of proper length to fall within the limits of a cover glass. In making such divisions it must be remembered that the ribbon expands somewhat in spreading, and allowance should be made for this. If the width of the face of the block is known the number of sections that can conveniently be placed under the cover can be determined by noting how many times the dimension will go into the length of the cover. It is desirable, also, to allow some space at each edge of the cover so that the sections will not lie too near its limits. Having determined the proper length of ribbon segment, take enough of these to occupy the width of the cover glass.

To prepare the glass slip for receiving the sections it is necessary to remove from its surface all foreign material. Commonly this is done by soaking a number of glass slips in battery fluid (p. 51). In place of this some technicians use acid alcohol. Do not touch the glass with any cloth that has not been washed in acid water. Paper towels are clean and convenient for drying. By these means the substances accumulated on the glass surface are removed and it is left chemically clean. *This is an absolute requirement.* As has been indicated previously, the effectiveness of cleaning may be tested by noting whether the albumen water adheres uniformly over the entire surface. If it should show any tendency to roll up into droplets it is a sure test that the surface is not perfectly clean and the slip should be returned to the battery fluid and left.

Upon the thoroughly clean glass slip is placed a quantity of dilute albumen water (p. 475). Upon this are arranged the segments of the ribbon placed so that the first section of the first segment is at the upper left hand corner. The succeeding sections follow each other as do the words in the lines of a printed page. When these segments are properly placed the glass slip is laid upon the table beneath the incandescent lamp which was used to melt the paraffin. At this distance the paraffin ribbon will probably not melt. If it is found that there is a tendency for it to do so the lamp should be lifted somewhat so that there is no danger of overheating the sections. At the same time the temperature should be very near to the melting point for the best results. As soon as the heat begins to affect the ribbons the sections expand and eventually reach a dimension equal to that which they would have in the absence of compression during sectioning. At this time the paraffin, previously showing white, becomes translucent so that against a black background it is hardly visible. *The requirement here is to secure complete extension of the ribbon.* Any irregularities in the ribbon, if not removed at this stage of the process, will remain permanently as ridges or waves in the sections.

When the ribbon is completely extended, the preparation is cooled, the excess albumen water is drawn away, and the ribbons are manipulated by

means of pieces of absorbent paper so that they lie perfectly straight and parallel on the slip with the sections of different rows in lines at right angles to their length. When this has been accomplished, absorbent paper is used to draw off the excess fluid and the mounts are placed in covered receptacles so that they may not be exposed to the dust.

It is convenient at this time to mark each of the slides permanently with a distinguishing number, as indicated under "Labeling" (p. 26).

13. Drying is a process of removing the remaining water by heat. The slips with the sections upon them are placed in some warm place, such as in the neighborhood of the incandescent lamp, where they are allowed to dry completely. It is best to let them rest in this position for several hours—overnight if possible. At the end of that time the water is completely removed from beneath the sections and they should then lie intimately in contact with the glass surface. Whether they do or not can be determined by turning the slide over and having the light from the window reflected from the surface of the section next to the glass. If it is in immediate contact it will show its complete surface without interruption. Should it, in whole or in part, be separated from the glass by an air space, this will show as a silvery white area. If there is very much of this separation the section will slip away from the glass after the removal of the paraffin. When spreading has been so incomplete that the sections are nowhere adhering to the glass they may sometimes be refloated on dilute albumen water and further spread, after which they are dried as previously directed. Completion of the spreading process should find the entire series of sections directly adsorbed to the glass surface.

In preparation for the succeeding steps a definite arrangement of materials is desirable. Accordingly a brief account of the equipment will be given. Commonly the sections are mounted on glass slips 25 mm. × 75 mm., but embryological mounts are often made upon slips as large as 50 mm. × 75 mm. The size of the vessels employed is accordingly to be determined by the dimensions of the slips. There are two general means utilized for carrying the preparations through the various solutions. One of these is to place them in a proper sized glass box with divisions for holding the slides upright, and allow them to remain in this vessel, to which the different solutions are successively added and withdrawn. The other is to lift the slides from one vessel to another containing appropriate media or reagents. If large numbers of slides of a similar character are carried through at one time the former method is better because it avoids handling them individually.

If, on the contrary, a small number of slides or those of diversified character are to be handled, it is necessary that they be treated individually and under these circumstances the more convenient way is to transfer them from one vessel to another. For this purpose a jar of distilled water and a series of alcohols, each contained in its separate tall Stender dish, are

arranged in order from 30 per cent to 95 per cent, followed by a Stender dish containing a mixture of equal parts of 95 per cent alcohol and xylol and finally a jar of pure xylol. Since the slides are run both up and down the series, there is some advantage in having two jars of xylol, one used in the ascending series and the other in the descending. If the sections are to be stained on the slide there should be Stender dishes with appropriate solutions.

14. **Decerating** is the process of removing paraffin from the sections. Until the sections are quite firmly adherent to the glass the paraffin is necessary to support the tissue, but when thus attached they no longer require the paraffin, and, since it interferes with the further operations of staining and mounting, it must be removed. This is done most conveniently by plunging the entire slide into a jar of xylol, which almost at once dissolves the paraffin. If large numbers of similar slides are carried through in the glass boxes, the only difference will be that the fluids are successively applied to the slides in the one container. *After the removal of the paraffin the sections must never be allowed to dry.*

15. **Preparation for Staining.** There are no stains commonly used which are dissolved in the medium employed to remove the paraffin, so the xylol must be displaced before staining is possible. The first step then is to dissolve out the xylol with 95 per cent alcohol from which the sections are directly transferred to the stain, if it is an alcoholic one. In case the stain is aqueous, as it commonly is, it is necessary to hydrate the sections by running them down through graded alcohols from 95 per cent.

Transfer the slides successively into the jars containing a mixture of alcohol and xylol and down through the descending series of alcohols to water. The slides should remain in each of the strengths of alcohol only long enough to secure the substitution of that strength for the one preceding. This is indicated by the uniform appearance of the fluid as the slide is lifted from the jar. So long as there is a difference between the strength of the alcohol in the sections and that of the solution, diffusion currents will appear. In proceeding down the series it is not necessary to be particularly careful about drawing off the alcohol of the higher concentration since it only serves to increase the percentage already present. However, in proceeding in the reverse direction, in order to avoid diluting the alcohols of higher concentration it is best to remove the excess fluid with absorbent paper. A paper towel to which the slides are touched is convenient for this purpose.

16. **Staining.** This is the process of increasing the visibility and contrast of cell and tissue parts by their differential reactions to dyes (for details relating to staining see p. 462). According to the method of their application stains fall into two classes, first, the progressive stains which are operated by leaving the sections exposed to their action until the desired degree of coloration is reached; and, second, regressive stains

which are allowed to act until overstaining is accomplished, after which the desired degree of differentiation is brought about by removal of the excess coloration. It will serve the purposes of the present outline to indicate how representative examples of these two methods may be employed.

Common stains are primarily nuclear stains and of these hematoxylin is probably the best. It may be applied in either of the two ways indicated. As an example of direct staining we may consider the application of Delafield's (p. 468) hematoxylin or Mayer's (p. 469) haem-alum.

The sections with the paraffin removed and hydrated are ready to be placed immediately in the staining agent. This is preferably contained in a convenient-shaped glass vessel so that the slides remain in a vertical position. Into this is placed a dilute solution of Delafield's hematoxylin. As a general principle it should be recognized that the more dilute the solution the longer the operation and more precise the result of staining. If rapid effect is desired the stain should be used in a more concentrated form. The only way that the effect of the stain can be determined is by examination of the sections under the microscope. Only in exceptional instances, for specifically indicated materials, is it possible to give the exact time for the staining operation. It is suggested that as a trial period the sections of liver be stained with one-fourth strength Delafield, 5 to 10 minutes. When by examination it is found that the desired structures are properly colored, the operation of the stain may be terminated by putting the slides in tap water for at least ten minutes, where the excess of stain is removed. In the event of over staining, this excess may be reduced by placing the sections for a short time in acid alcohol. The color of the stain depends upon its acidity, being red if acid, or blue if alkaline. Since tap water is slightly alkaline, washing in this medium gives an agreeable blue. Washing should always be thorough after staining and differentiating in order to secure permanence and precision of the stain. Mayer's haem-alum, although not so generally used as Delafield's mixture, produces a more exact and delicate result, even when used undiluted.

As an example of the regressive stain, the iron hematoxylin method of Heidenhain may be instanced. In this process the sections are first mordanted for a period of from two to twenty-four hours in a 4 per cent solution of ferric alum. They are rinsed for a sufficient length of time to remove the excess iron alum solution and are then placed in a ½ per cent aqueous solution of hematoxylin where they remain for a period of time approximately equivalent to that employed in mordanting them. At the end of this operation they are completely black, showing practically no differentiation whatever. To accomplish the desired degree of destaining they are rinsed quickly (thirty seconds) and returned to the mordanting solution which now acts as a differentiator. Gradually the hematoxylin is dissolved out, beginning with the cytosome and proceeding to the nucleus. The last substance to lose the stain is the chromatin. It is possible by this method

to secure the most extreme degree of selectivity between different portions even of the chromatin, and it is one of the most valuable of fine cytological methods. The quality of the stain is improved by frequently rinsing the slides in water as they are examined under the microscope during the process of differentiation. The longer operation of staining gives a greater range of coloration which can be thus extended from the nucleus to the cytosome, bringing out such structures as centrosomes which are lost in the more rapid process. In some cases satisfactory results can be secured by mordanting and staining for periods within an hour.

After differentiation is complete the excess of iron alum must be very carefully removed by washing an hour in running water. The resultant color of the structure depends in some measure upon the age and quality of the hematoxylin solution. Fresh solutions give a bright blue effect, while those that have been repeatedly used range through black to a rusty green color. The character of the wash water also has an effect upon the color. At the end of either progressive or regressive methods with hematoxylin we have a result showing the nucleus more densely colored than any other portion of the cell, while within the nucleus the chromatin is strongly distinguished by its greater affinity for this coloring agent.

17. Counterstaining is the process of adding one or more additional stains, which, by contrast of color and selectivity to the primary stain, emphasize distinctions between cell and tissue elements. In some cases it is desirable to thus differentiate the parts of the tissues or cells by the selective influence of different kinds or colors of stains. If hematoxylin has been used as a nuclear stain it is a common practice to emphasize the cytoplasmic structures by means of such counterstains as eosin or Congo red, which, both in color and selectivity, contrast with hematoxylin. The easiest way of applying such counterstains is to have an alcoholic solution of them and pass the sections through it in the process of dehydration. Care should be exercised not to vitiate the bright blue color of the hematoxylin by admixture of the eosin, which is so general a stain that it will take in any part of the cell. In most instances the use of a counterstain is of doubtful value, since there are rarely revealed structures which are not sufficiently differentiated by the primary nuclear stain.

A counterstain may be applied to the piece of liver as indicated.

18. Dehydration. Most staining agents are aqueous, and since the mounting medium is commonly a balsam, the water must be removed by the usual method of dehydration, running the sections up through grades of alcohol into 95 per cent and even absolute. While it is perhaps not necessary always to take this precaution, in exceptional instances the rapid transfer of sections from water to 95 per cent alcohol may injuriously affect them. A slight expenditure of time in running up through grades of alcohol will obviate this and as a matter of routine it seems to be worth while to employ

the gradual method of dehydrating except where certain stains are too readily removed by the alcohols.

19. **Clearing.** The sections, after having been dehydrated, are prepared for enclosure in balsam by passing them through some clearing medium, commonly xylol. It is necessary to obtain a complete removal both of alcohol and of the water which preceded it; otherwise the sections will become clouded when placed in the balsamic mounting medium. Clearing with one of the essential oils in order to avoid the condensation of water by the evaporation of the xylol is helpful. A rapid but thorough rinsing out of the clearing oil by xylol should occur.

20. **Mounting** is a process of enclosing the prepared sections in some medium which will preserve them indefinitely under suitable conditions for microscopical observation. The slide is removed from the clearing medium, the excess of this being wiped from the surface with absorbent paper. Upon the center of the group of sections is placed a sufficient amount of gum damar which has been dissolved in xylol, to occupy the space between the cover and the slip. Only experience will indicate how much of this should be used, but endeavor not to add an excess of balsam because it makes a difficulty later in cleaning the slide. On the other hand, see that there is no space left beneath the cover not filled with the mounting medium. A perfectly clean cover glass is held by forceps and gently lowered obliquely upon the surface of the mounting medium. If the contact is made too rapidly, small air bubbles are almost certain to be enclosed. By careful manipulation, however, the cover can be placed upon the balsam so as to completely exclude all air. The cover is then lightly pressed down so that it is brought into contact with the sections beneath. It is quite undesirable, especially for high power observations, to have any excess quantity of balsam between the section and the cover glass. Any considerable surplus of balsam should be carefully wiped off with absorbent paper after which the slides are ready for the next process, that of drying.

21. **Drying** is the process of removing the solvent from the resinous mounting medium so that the sections are left enclosed in a solid substance. In order to remove the excess of the solvent from the mounting medium the slides are placed in a horizontal position in some warm place where evaporation is facilitated. They should remain there until the balsam has become thoroughly hard, so that pressure upon the cover glass will not cause it to move over the sections. Any excess of heat, however, should be avoided, since it may injure the tissue, while the too-rapid evaporation of the solvent will cause open spaces beneath the cover glass and may even injuriously affect the quality and color of the mounting medium.

After the damar is thoroughly hardened, the excess is removed from around the edges of the cover by scraping. One should be careful to avoid contact between the knife which is used for this purpose and the edge of the cover, which may thereby be broken. Finally, the remaining balsam

outside the edges of the cover is removed by dipping the slide in xylol and wiping it carefully with absorbent paper or cloth.

22. Labeling. If any number of slides have been carried through together each of them should bear a distinguishing number. Preferably this is the number which marks the lot, supplemented by the number of the slide in the series. The best way to indicate permanently the source of the material is to scratch its number upon the surface of the glass slip with a diamond. In large collections which are completely catalogued this is a sufficient method of labeling individual slides, but in cases where these are consulted by many people it is helpful to have in addition a label which indicates the nature of the material and the character of the processes through which it has passed. Commonly this is done by writing or printing the data desired upon paper labels of proper size and then attaching these by means of gum to the ends of the slides. It is best to have a regular system of labeling, and most workers place the main label at the left end of the slide. Upon this there may be a complete record of the source and character of the material, the nature of the processes through which it has passed, the date, and the name of the preparator. A complete preparation of this sort is properly designated as a microscopical slide. Unfortunately this term has become confused with the term "slip" which should be applied to the piece of glass upon which the object is mounted.

23. Storing. There are various methods for permanent storage of slides, with characteristic advantages and disadvantages. The most common method is to place the slides in wooden boxes bearing racks into which the ends of the slides fit. These are of various sizes, but commonly one holding 25 slides is used. There are other boxes, usually made from cardboard, in which the slides are laid horizontally protected by a cover which falls over them.

One of the very best methods is a recent contrivance. This is an envelope without a flap, made of heavy paper, such as "Paperoid."* These envelopes are 3×5 inches in size, sealed at the ends, and divided into four compartments by uniting the front and back with staples. The upper portion of the front is cut off $\frac{1}{2}$ inch shorter than the back so that when the slides are inserted into the compartments the label is exposed. If labeling has been done with a diamond, this provides sufficient space so that the designating numbers are visible when the group of four slides is examined. These containers, holding four slides each, may be inserted vertically into any of the devices used for filing 3×5 library cards and may there be combined with records and drawings, pertaining to them individually, which have been placed upon library cards of the same dimensions. This system has many advantages, since it occupies only a limited space, leaves no vacancies, protects the slides thoroughly so that they may be shipped without any danger of breakage, and in the event that the container is dropped on

* Hance, R. T. *Anat. Rec.*, 1924, xxviii, No. 5.

the floor, protects them from injury. It has the very great advantage of extreme flexibility, providing a means not only for storing a complete collection readily, but for withdrawing all of the slides relating to a particular species or subject without necessity for sorting. Altogether it is the best arrangement that has been so far devised for storing and consulting collections of slides.

If slides are used by more than one person their loss may be prevented and the convenience of consultants served by inserting into the space from which each slide is removed a card of the same size upon which is recorded its number, the date of withdrawal and the name of the person having it. To make such an entry requires little time and trouble and will often save valuable specimens.

II. Collodion Method

1–6. In the application of the collodion* method the steps follow in order as outlined in the paraffin method from 1–6 inclusive. Dehydration must be complete since the collodion is dissolved in the mixture of absolute alcohol and ether in equal parts. Treatment in absolute alcohol is therefore required.

7. Since the material goes from absolute or from the alcohol-ether mixture directly into the infiltrating medium, item 7 of the paraffin method "Clearing" is omitted.

8. **Infiltration.** This is accomplished without the application of heat by placing the thoroughly dehydrated material directly into a thin syrupy solution of trinitrocellulose. The time required for infiltration depends upon the size and character of the pieces of tissue. The object to be attained is to displace the absolute alcohol by thin collodion. When this has been done the next thing is to secure a gradual evaporation of the solvent so that the tissue becomes infiltrated with a thicker and thicker concentration of the collodion. This stage may require many days or even weeks if the specimen is large. It is necessary to provide so gradual an evaporation that concentration is uniform throughout the mass. When the infiltrating medium becomes concentrated almost to a gel the material is ready for the next process.

9. **Imbedding and Hardening.** The piece of tissue is brought into a paper box or vessel of appropriate size filled with thick collodion. This is then put aside under a small bell jar and evaporation allowed to continue somewhat more rapidly until the syrupy fluid has become so hardened that it will retain an impression of the finger nail. Care must be exercised that evaporation is not so rapid that the concentration of the collodion outside of the tissue is greatly different from that within. A final hardening

* Collodion is a solution of a trinitrocellulose made from cotton. Other forms of the compound recently developed have the special names, celloidin, parlodion, etc. Collodion is here used in a generic sense.

of the collodion is accomplished either by placing the block in 80 per cent alcohol or by exposing it to the vapors of chloroform. Sometimes hardening is secured by treatment with chloroform followed by 80 per cent alcohol. As a result of the treatment the collodion is now of such a consistency that it will readily section. If the piece of tissue is small enough infiltration and imbedding may conveniently be carried out in an appropriate sized shell vial from which the cork is occasionally removed for brief intervals to regulate the rate of evaporation. When the medium is sufficiently stiff the object is oriented to be cut parallel with the bottom of the vial and a little later the mass is loosened from the vial. When stiff enough finally it may be removed from the container.

10. **Blocking and Trimming.** The mass of hardened collodion with the contained tissue is mounted upon a proper support with thick collodion which is set after a few minutes drying by immersion in 80 per cent alcohol for three or four hours. Fiberoid blocks are convenient for this purpose. Small floor tiles are also used. It is not necessary, as in the case of paraffin, to trim the block to any particular shape so that the only requirement here is to remove any excess of the mass about the tissue.

11. **Cutting.** Instead of cutting the sections with a dry knife placed at right angles to the direction of movement, the collodion sections are cut with a wet knife adjusted at an extreme angle to the direction of movement. Cutting is facilitated by having a conveniently placed vessel which drops 70 per cent alcohol upon the knife's surface at such a rate that there is always a small pool of fluid at the point where the section comes off. The manner of sectioning varies with the microtome used, but should be accomplished with a firm uniform movement. The sections are most conveniently handled by a moistened camel's hair brush. If they are to be mounted serially they are moved into position successively as they are cut until a sufficient number has been accumulated in proper arrangement for mounting. They are removed to the clean glass slip by placing a thin piece of tissue paper over them and pressing this down until the sections adhere. By carefully sliding the piece of paper and the adhering sections off the knife they can then be transferred to the glass slip. Press the sections into contact with the glass surface by rolling them with some cylindrical object, after having interposed several layers of absorbent paper, when they will adhere sufficiently so that the paper may then be removed. The sections may be caused to adhere permanently to the glass by pouring over them the vapor of ether which softens the collodion, or by action of clove oil which has a slightly solvent effect. Remove the oil after five minutes with 95 per cent alcohol. Mayer's albumen applied in a very thin layer to the glass will also cause the sections to adhere. After the sections are arranged, coagulate the albumen with 95 per cent alcohol.

12–15. These steps of the paraffin method are omitted since the sections are spread by the above-described operation. Drying is omitted

because the sections already adhere to the glass and because it is not possible to dry the collodion sections without injuring them. The imbedding medium is not removed at any time in the collodion method.

16. Staining. Staining may be done after the sections are attached to the glass as in the paraffin method, but more commonly for histological work the material is either stained in toto or the individual sections are floated in the stain and thus prepared before attaching them to the glass. This is not feasible, however, in case of serial sections. The staining of the attached sections is carried on according to the manner described in the paraffin method.

17–23. These steps are performed as described in the paraffin method.

Modifications of the Collodion Method

Gilson's Rapid Process. It is sometimes necessary to secure sections by the collodion method more rapidly than is possible with the usual procedure. The objects are infiltrated with collodion while heat is applied by dipping the tube containing the material into melted paraffin. Here the collodion boils and produces a very rapid evaporation of the solvent. After it has reached a consistency which will just permit it to be poured from the vessel the specimen is removed and placed upon a block of hardened collodion and is then surrounded by the thickened medium. This is then hardened in chloroform and cleared in cedar oil. After the object has been mounted on the microtome the sections are cut with a knife moistened with cedar oil. If the material has been stained in toto the sections are then ready to mount.

The Dry Cutting Method. This is somewhat similar to the method of Gilson but with slow infiltration. After the object has been infiltrated it is hardened as usual with chloroform vapor and is then placed in the mixture suggested by Gilson; that is, equal parts of chloroform and cedar oil. To this, cedar oil is added by degrees until the mixture is replaced by pure cedar oil. The block is then ready for cutting after being properly mounted. It is cut dry, requiring only that the knife be moistened with the cedar oil in order to prevent the sections from tearing. Sections are ready for mounting if they have previously been stained. In case it is desired to stain the material it will, of course, be necessary to run the sections down through alcohols until the proper concentration to meet that of the stain is reached.

The dry method has several advantages over the ordinary one. The sections being infiltrated with the oil are not so difficult to handle and the blocks from which they are cut may be preserved indefinitely because of the presence of the oil.

III. Freezing Method

This is a method not wholly adapted to precise results, but at the same time is one of very great convenience. It is rapid and easy of application and gives results sufficiently good for diagnostic purposes. It is, in fact, most often used by diagnosticians who are obliged to get information with regard to the character of a tissue as rapidly as possible. It is also useful in studies of cell constituents when ordinary methods would destroy or injure the substance to be studied.

Perfectly fresh tissue, just removed from the animal, may be placed upon the freezing microtome and sections cut from it as soon as it has been frozen. More frequently, however, the material is fixed and is then placed

in a medium which is better adapted to sectioning than plain water ice. Of the fixatives most commonly used 4 per cent or 5 per cent formol is best. If there is need for haste, after fixation the material may be frozen in the fixative, but commonly it is better to wash this out and substitute for it a medium made by combining a saturated solution of sugar, 3 parts added to 5 parts of mucilage prepared by dissolving 60 gm. of gum acacia in 80 c.c. distilled water. The tissue is infiltrated with this medium and then removed to the holder on the microtome where it is covered by a quantity of the mucilage. It is frozen in this fluid and sections are then cut.

Frozen sections may be cut with an ordinary microtome, but more frequently a special piece of apparatus attaching directly to a carbon dioxide cylinder is used. In this case a knife in the form of a chisel is employed to cut the sections. As in the case of other section methods the skill required can be gained only by practice but there are some suggestions which may be of service. In order to prevent the knife from melting the mass as it passes over it, it should be kept cold. This also prevents the sections from sticking to the knife and becoming torn. Speed is an essential requirement in sectioning with this method. Sections are handled with a camel's hair brush like the collodion sections.

After the sections are cut the gum solution is removed by immersion in water and then they are ready for staining. The common method is with Delafield's hematoxylin which may be followed by eosin for a counterstain. Following this the sections are treated as in the other methods, mounting them finally in damar.

IV. Smear Method

The essence of the smear method is to form a very thin film of the tissue upon a glass surface so that the elements are individually distinguishable. This is accomplished in three ways. The first is by placing between two clean cover glasses a small quantity of the material to be treated. The cover glasses are then pressed together so as to dispose the material uniformly between them. If the substance is a fluid like blood no pressure is required, but in most other instances a certain amount is necessary. This is a delicate point in manipulation and the extent of pressure can be determined only by experiment. The end to be aimed at in most cases is to manipulate the materials so as to separate the cells and to avoid crushing them. In some instances, however, it is desirable even to rupture the cell in order to distribute its formed elements, like the chromosomes, so that they may be more readily observed. Except in the case of fluids, it is impossible to secure absolute uniformity in the thickness of the film and in some respects this is an advantage because various degrees of extension of cell contents may be found in different regions of the preparation.

Assuming that the proper distribution of the elements has been obtained, the next step is to slide the two covers apart rapidly and uniformly. Two

precautions should be observed; First, to separate the covers exactly in the plane of their contact,—they should be slid apart and not pulled apart. Second, the movement of separation should be uniform. If it is not there will be areas of different density according to the speed with which the separation has proceeded. Even with the best of manipulation, however, it is rarely the case that the films on the two covers are equally good. If it should happen that the tissue is of such character that there are residual masses of considerable thickness it will be desirable to remove these after fixation.

The subsequent treatment of the film depends upon the character of the material. In the case of blood the films are fixed by drying and there are other instances where this is possible. Some materials, however, are entirely ruined by allowing any evaporation to take place and in these cases the films should be dropped immediately upon a fixing fluid after separation of the cover glasses. What will happen to any given kind of cell can be determined by experience alone. As an example of the variation of behavior in this respect it may be stated that the cells of Hemipteran testes are quite uninjured by drying while those of the Orthoptera are completely ruined.

Another method of obtaining a film is exemplified in the ordinary process of making blood smears. Here a drop of blood is picked up on the end of a clean glass microscopical slip and this is at once applied to the surface of another slip. By a uniform and fairly rapid movement the blood is distributed over the surface of the second slip by moving the first along its length. There are several precautions which should be observed in this procedure. The edge of the slip chosen to carry the drop of blood should be perfectly straight so that it will lie in contact evenly with the surface of the other upon which the blood film is to be formed. Also it is most desirable that the second slip have as uniform a face as possible. If there are variations in these two matters the film will be of unequal thickness. Likewise it is necessary in spreading the film to proceed with a uniform movement; otherwise inequalities in thickness will be produced. The best method of operating is to lay the second slip upon the surface of the table, applying to it, near one end, the one with the blood, at an angle of about 45°. Hold the glass slip carrying the blood between the thumb and first finger, and support the hand on the table by the tips of the remaining fingers. In this position the movement of the carrier can be made uniform in pressure and rate. (For a detailed account of the use of this method see the section on Blood, p. 244.)

A variant of this second method of smearing may be employed in the case of such tissues as testicular material where it is desired to get films of the germ cells. Where this is possible a film is produced by taking a piece of the tissue with a pair of forceps and wiping it over the surface of the glass slip. In this way a layer of the contained germ cells is left behind. This is a method which has been used very successfully in the case of Hemipteran

germ cells, gland cells, bone marrow, etc. A third method used with plant cells is described on p. 116 Chapter III. Films obtained in any of these ways, after fixation and proper washing, may be stained in the same way that sections are and mounted properly with damar.

The advantages of the smear method are that, with it, preparations may be rapidly secured in which the entire elements are preserved and often in such a form that details of structure are unusually well displayed. The disadvantages lie in the fact that normal relations of some elements are disturbed and, by reason of variations of pressure and drying, cell elements are differentially affected. Only when necessary should observations based upon smears be used unsupported by a study of the same material in sections.

A special refinement of the cover glass method, adapted especially to secure perfect films for blood studies is thus described by Isaacs.

Cover glasses which have been soaked in nitric acid over night or in warm nitric acid for about one-half hour, are thoroughly washed in running water. A funnel held upright and attached by means of a rubber tube to a faucet serves as a convenient washing device. The washed cover glasses are placed in 95 per cent alcohol and kept there until they are to be used. With a pair of forceps they are transferred from the alcohol to a wide mouth bottle containing either ether or acetone. From the ether or acetone they are transferred to a piece of filter paper and allowed to dry. With the forceps they can be placed on a piece of smooth writing paper lying on a flat table. To polish the surface, the finger is placed on the glass, and with firm pressure the cover slip may be moved back and forth over the paper. After the cover glasses have been polished, they should be brushed with a large grease-free camel's hair brush to remove any lint or dust. In this way the side touching the finger becomes covered with grease, but the side nearest the paper will remain grease-free for several minutes. The blood is drawn in the same way as for making blood counts and when a drop about 3 mm. in diameter has accumulated, one of the cover glasses is touched to it and a drop about 2 mm. in diameter is picked up. The cover glass is then placed in contact with another clean and polished cover glass and the drop allowed to spread until it has almost reached the edges of the glasses. While it is still very slowly spreading, the two cover glasses are drawn apart with a short, sharp snap. If the cover glasses are placed over each other so that the corners project, a single corner of each cover glass may be used as a place for holding them.

The blood films are allowed to dry at room temperature for at least three minutes and longer if time is available. If the staining process is begun before the blood is dry, the the blood will sometimes come off the cover glasses. The best results are obtained if the films are stained within twenty-four hours after being made.

V. Stretching Method

Certain membranes in the body, like the omentum, are best prepared by this method. A convenient way of securing such preparations is to use two rubber rings which fit one within the other. By stretching the membrane over the smaller ring and then passing the large one over the smaller, the tissue is tightly stretched. The membrane is then trimmed around with scissors and in this condition can be handled very much like a section. The subsequent treatment of the preparation depends upon the object in view. If it is to be stained and mounted in the ordinary manner the

series of steps indicated in the paraffin method would be followed with the exception of steps 8 to 15. Other material, like subcutaneous tissue, may be prepared by a modification of the method.

Subcutaneous Tissue Spreads. Loose subcutaneous tissue is found in any vertebrate just under the skin, between that and the muscles. It is usually taken from the abdominal or inguinal regions.

After the selected area is shaved, an incision is made in the skin and a very small piece of subcutaneous tissue is snipped off with scissors. This piece is placed on a cover glass and quickly spread out as flat and as thin as possible by means of two teasing needles, taking care, however, not to tear it to the extent of injuring the cells. It is an advantage for two people to work together in doing this, using two needles each. The cover glass is then floated on fixing fluid, the tissue side being down. All the above manipulations must be done quickly before the tissue dries, and it facilitates handling the cover glass if the teasing is done with the cover glass over a black background and supported by a Petri dish which is bottom side up. (Slider.)

There are certain other more rigid structures which may be mounted entire like these more delicate membranes. An object of very great value for microscopical observation, for instance, is the gill plate of certain amphibia, like Amblystoma. These structures have sufficient rigidity so that they may be handled like collodion sections and treated very much in the same manner. They do not require the stretching process but are treated, otherwise, essentially the same.

The advantage of a method of this character is that the structures are all preserved in their normal relations while at the same time one has almost the same conditions of thickness as is found in sections. In cases where cells are not cut through, however, the staining is in some ways less precise. However, when a regressive method like Heidenhain's iron hematoxylin is used, this is not a serious difficulty. In very thin membranes like the amnion, cytological details are well presented.

VI. Teasing Method

Certain fibrous tissues like white fibrous, yellow elastic, and muscle, are advantageously prepared according to this method. Before teasing it is desirable to fix in such a way as to get a differential action upon the interstitial substances. For this purpose dilute fixatives are commonly utilized so that a partial maceration precedes the actual act of teasing. After the material has been properly fixed, it is taken in small pieces, either on the glass slip where it is later to be mounted, or in a watch glass, and combed out by the use of two dissecting needles. Care should be exercised not to break the fibers across, but rather to comb along their length so that the cement substance is removed, leaving the fibers free at their ends.

By a sufficient degree of maceration the real act of teasing is much reduced in extent, and it is often possible to almost entirely dissociate the elements previous to their mechanical separation. It is sometimes advan-

tageous to stain the material before teasing, but if this is not done a stain may be dropped upon the material as it rests on the slip or in the watch glass, and its action watched under a microscope. The further operations of differentiating, dehydrating, and clearing are also accomplished preferably on the slip where the material is to be mounted.

Difficulties in teasing that may be encountered are largely due to the lack of proper treatment in fixing, and it is often better to take additional time in the earlier steps of the process rather than to try to separate the tightly joined fibers by force with the needles. In general, what will be secured by an operation of this sort is a number of masses of material from which free elements project more or less. The limitations of this method are obvious and while it is useful in certain cases, it is inferior to many of the others as a general method.

VII. Grinding Method

This method has very special applications which will be considered in detail under the topics of bone and teeth. It is a physical method which involves the use of abrasives upon hard structures. Appropriate-sized pieces of tissue are fastened by cement to the glass upon which they are to be mounted, and then by grinding and polishing, the thickness is reduced sufficiently so that the light will pass through. After washing, cleaning and drying the surface the specimen is mounted in balsam beneath a cover (p. 268).

VIII. Macerating

All the preceding non-section methods of making microscopical preparations are physical in character, but maceration depends upon chemical action. As has been explained, it is made possible by the differential effect of weak fixatives upon interstitial or cement substances. An appropriate piece of tissue is placed in the weak fixative and allowed to remain sufficiently long for the reagent to dissolve the interstitial substance and then, upon shaking in a bottle, the elements may be freed from each other and made ready for further treatment. This will depend upon the fixative employed, but assuming that any ordinary one has been used, the next step would of course be the removal of the excess fixative by washing. The various steps of the paraffin method can then be followed, leaving out those from 8 to 15.

The several processes can best be performed in a small shell vial, because the elements of the tissue, either by gravitation or by centrifuging, may be brought to the bottom of the tube after each treatment, and the supernatant fluid can be withdrawn and that required for the next step added. It is even possible, and in some cases very desirable, to carry the cells clear through into the balsam where they may be kept indefinitely and removed with sufficient medium to the slip for mounting.

The advantage of the maceration process is that entire elements are obtained. The disadvantage is that because of the use of a weak fixative the elements of the cells are not nearly so accurately preserved as they would be with a normal fixation. In most cases, therefore, if it is desired to obtain detailed information with regard to the structure of intracellular elements, the maceration process should not be employed.

PART II

SPECIAL METHODS

CHAPTER II

METHODS FOR THE STUDY OF FRESH MATERIAL

Physical Agents: Microdissection, Microinjection 39. Chemical Agents 74. Vital stains 74. Supravital stains 81.

PHYSICAL AGENTS: MICRODISSECTION, MICROINJECTION

By Robert Chambers

Free-hand Manipulations 39. Micrurgical technique 42. Applications of micrurgical technique 68.

The micrurgical* or micromanipulative technique involves procedures which fall into two groups: (I) methods for operating with the free hand under low magnification and (II) the more strictly, so-called, micrurgical methods which have been developed for the purpose of operating under low and high magnifications of the compound microscope.

I. Free-hand Manipulations

Under this heading may be grouped methods for operating under comparatively low magnifications. Mechanical devices involving the use of ball-and-socket and sliding movements can be readily arranged for particular purposes and descriptions of some have appeared from time to time, especially in the older literature on microscopy and microscopical methods.

1. Dissection. Many methods have been improvised by numerous investigators for the free-hand dissection of minute objects in the field of the dissecting and compound microscope.

These methods depend not only upon the ingenuity and manual dexterity of the operator but also on the type of implement used. Slivers of glass mounted on handles and specially sharpened knives and needles have proved to be serviceable. For cutting purposes a useful implement, suggested by Fry,† is a long, tapering glass hair which is drawn over the object, e. g., an Echinoderm egg, in a drop of water on a glass slip (Fig. 1, F).

The hair is the end of a glass capillary about 0.2 mm. in diameter and 5 mm. long. The capillary is drawn out on the end of a 6 mm. glass tube which serves as a handle (Fig. 1, A–E). The hair is made in a microflame in a manner similar to that for making microneedles as described on page 52. The capillary melts as it is brought near the flame, and, if pulled at the right moment, will separate as it melts. This frequently results in a long, flexible hair which tapers gradually to a tip that may be but a micron in diameter (Fig. 11, e, page 55). The capillary shaft is bent in the flame at an angle of about 150 degrees.

The cutting of a cell is performed on a clean, grease-free slip on the stage of a dissecting microscope. With the hair, it is pushed near the edge of the drop where the decreasing depth of water slightly flattens and holds it. There it can be rolled into the desired position and cutting operations can be performed with surprising accuracy.

* Micrurgy (micros, small; ergon, work) is a term introduced by Péterfi (see footnote, p. 43) to denote micromanipulative technique in the field of the microscope.

† Fry, H. J. *Anat. Rec.*, 1924, xxviii, 371.

Chabry's method* for destroying one or more blastomeres of a segmenting ovum involves the use of a spring carrying a delicate glass needle held taut by a trigger. When the trigger releases the spring the needle-tip

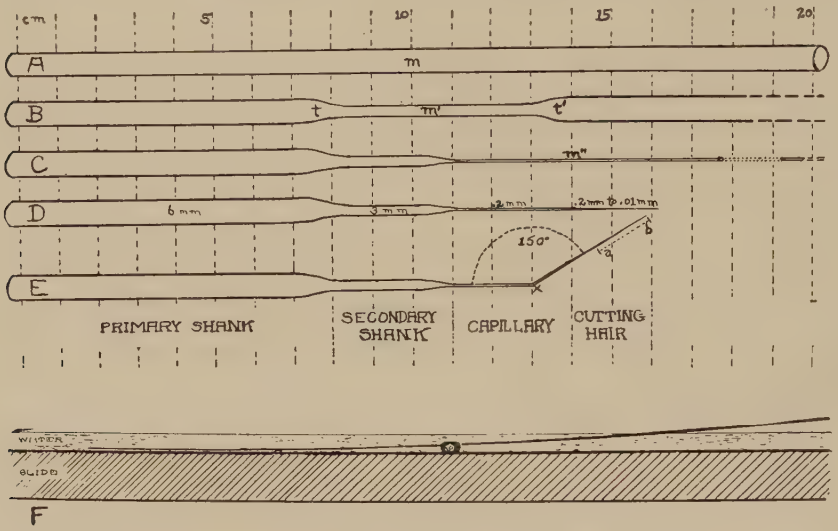


FIG. 1. Fry's method for making glass hairs for cutting cells. A to E, Stages in needle making. A, primary shank, 6 mm. tubing, for two needles, on which secondary shank is to be drawn at *m*. B, once-drawn tubing for two needles, in which capillary tubing is to be drawn at *m'*. C, twice-drawn tubing for one needle, in capillary stage, in which cutting hair is to be drawn at *m''*; dotted lines indicate mid-region of capillary where it has been broken off; tip of capillary of other needle is shown at the right. D, thrice-drawn needle, with completed point, ready for bending. E, completed needle; portion of cutting hair shown in F, is indicated by the line a-b. F, diagrammatic view of cutting operation.

flies into the field of the microscope and punctures the blastomere of an egg previously placed there. A mechanical device controls the distance that the needle will travel.

Tschachotin's contrivance† for puncturing a cell is a needle holder fastened to the side of the objective of a microscope. A curved glass needle is mounted in the contrivance and, by manipulating certain screws, its tip is brought into the focal field of the objective. Operations are performed by bringing the cell into position by means of the mechanical stage and then simply raising and lowering the tube of the microscope.

2. Injection. Expansion, caused by heat, is the basis for the following method devised by Knower‡ for injecting the vessels of small embryos, etc.

A bulb is blown on one end of a capillary glass tube (Fig. 2, 1–4). The device is made as follows: A glass capillary is drawn out in a flame and one end is melted (Fig. 2

* Chabry, L. *J. d'anat. physiol.*, 1887, xxiii, 167.

† Tschachotin, S. *Ztschr. wiss. Mikrosk.*, 1912, xxix, 188.

‡ Knower, H. McE. *Anat. Rec.*, 1908, ii, 207.

1–3). It is then removed from the flame and the molten end converted into a bulb by blowing quickly through the capillary tube (Fig. 2, 4). The size of the bulb depends upon the amount of the molten mass on the end of the capillary. A serviceable size of bulb is

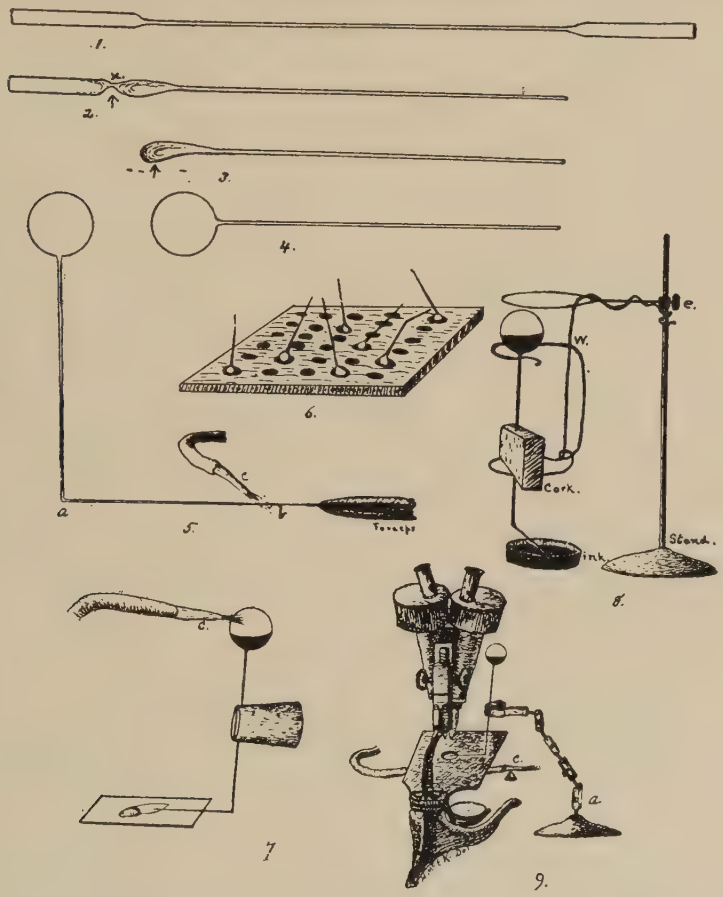


FIG. 2. Knower's method for making capillary glass bulbs for injecting small blood vessels. For description see text.

one ½–1 in. in diameter. A bend is now made in the middle of the capillary and a slender tip produced by drawing out the end over a minute gas flame (Fig. 2, 5). The tip may be clipped with scissors to the desired size. The fine tip is inserted into the injection fluid, e. g., India ink, and the bulb warmed, (Fig. 2, 8). Air will be driven out of the bulb which, on cooling, will partly fill with the fluid. The tip of the capillary is then inserted into the desired blood vessel under a dissecting microscope and the injection performed by warming the bulb (Fig. 2, 7 and 9).

Another good method is an adaptation of the Chambers' microinjection apparatus modified by Brown* for injecting the blood vessels of a chick embryo under the dissecting microscope (Fig. 3).

* Brown, A. L. *Anat. Record*, 1922, xxiv, 295.

The apparatus consists of an ordinary 2 c.c. glass syringe, A, which fits into the adapter, B, of a hypodermic needle, the tip of which is fastened with shellac or de Khotinsky cement into one end of a long, coiled piece of slender, soft, brass tubing, C, into the

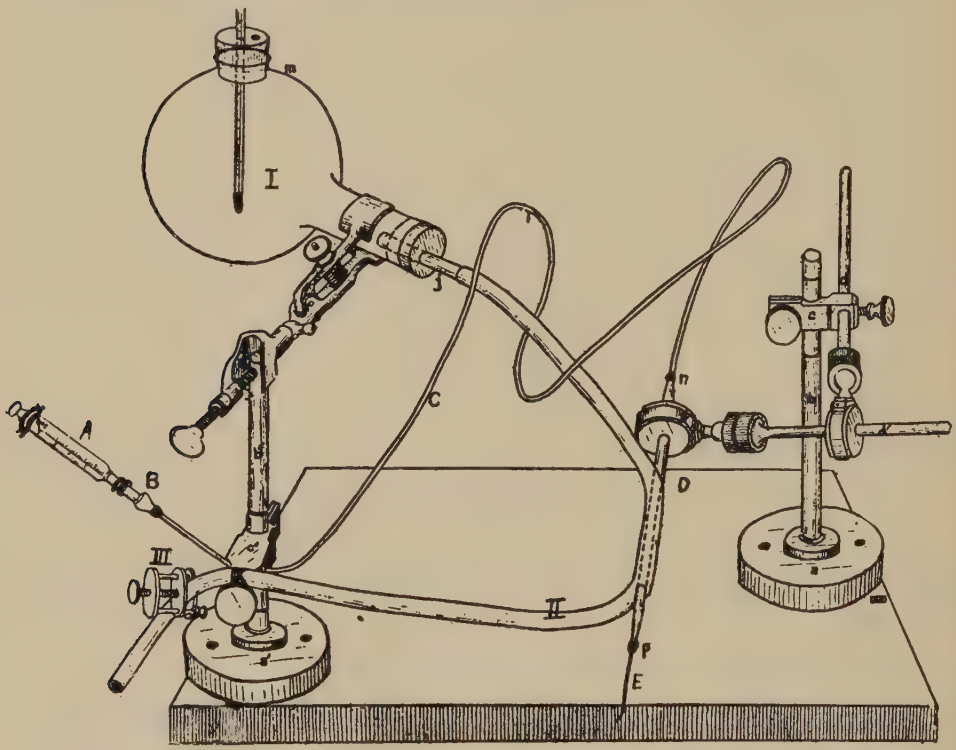


FIG. 3. Brown's method for making cold or warm injections into small blood vessels. For description see text.

other end of which is cemented, at N, a four inch piece of ⅛ inch glass tubing, D. The glass tube is drawn out at its free end into a slender shank with a bore having a diameter of about 1 mm., into which the injection cannula, E, is to be sealed with de Khotinsky cement. The cannula is a glass capillary, the tip of which is made by momentarily heating the capillary over a microburner and pulling it out just as it softens. The tapering tip may be broken off to form a pipette of the desired size. The support for the injecting device is a ball-and-socket contrivance which may be built out of two Leitz dissecting stands.

A warm injection can be accomplished by arranging a flow of warm water to heat the injection fluid in the glass tube which carries the cannula. The glass tube is thrust through the wall of a ½ inch rubber tubing, II, in two places. The upper end of the rubber tube is connected with a flask of hot water, I, and the lower end, which serves for the outflow, is shunted off to one side with a screw clamp, III, to regulate the flow.

II. Micrurgical Technique

Under this heading are included devices intended more strictly for work under the high powered objectives. In the instruments constructed

so far the three dimensional movements imparted to the needles or pipettes are practically at right angles to one another.

The micromanipulator is an instrument which is either clamped to the stage of a microscope or is mounted on a pillar in front of or to one side of the microscope. The needles or pipettes are mounted in special carriers on the instrument and project over the stage of the microscope into the field of the objectives. For comparatively low power work the needles can be placed so that their tips project down toward the stage of the microscope between the objective and the object. This method has been used for inserting pipettes into renal corpuscles and tubules and into blood capillaries.

When high powered objectives are used the operations must be performed with the tips of the needles or pipettes bent up and projecting into a hanging drop suspended from a coverslip which serves as the roof of the moist chamber (Fig. 3, d). The lack of any obstacle between the coverslip and the objective permits inspection of the operations with the highest magnifications of the microscope. Sufficient illumination can be obtained by the use of special substage condensers having a free working distance commensurate with the height of the moist chamber.

1. **Micrurgical Instruments.** There are three forms of micromanipulators in use at present, Taylor's,* Péterfi's† and Chambers'.‡ Taylor's instrument, (Fig. 4), is manufactured by the University of California and is based on the same principle as that of the original Barber Pipette Holder.§ Taylor's instrument, however, is far more stable and consists of two massive pillars mounted on a base which carries the microscope. It depends upon its mass and upon the extensive hand-ground surface of contact for the accuracy of its movements which are produced by an arrangement of three metal blocks, sliding in grooves. Péterfi's instrument (Fig. 5), manufactured by Carl Zeiss, consists of either two or three pillars mounted on a common base. It is much lighter than Taylor's and depends for its movements on both sliding and rocking devices. Chambers' instrument (Fig. 6), manufactured by E. Leitz, also consists of two or more pillars mounted on a common base. It is of about the same bulk as that of Péterfi's but depends for its fine movements upon the spreading apart of bars of rigid metal connected at their ends to form a Z-like figure by resilient metal acting as spring hinges. The tips of the needles of Chambers' instrument move in arcs of large circles. Under the microscope the curvature of the arcs is not appreciable but this feature somewhat limits the position of the instrument in its spatial relation to the stage of the microscope.

* Taylor, C. V. *Univ. Cal. Publ. Zool.*, 1925, xxvi, 443.

† Péterfi, T. Handb. microbiol. Technik., 1923, 2471. *Handb. biol. Arbeitsmethoden* (Abderhalden) Abt. v, ii, 479.

‡ Chambers, R. *Anat. Rec.*, 1922, xxiv, 1. (Reprinted in *J. Roy. Micr. Soc.*, 1922, 373.)

§ Barber, M. A. *Phillip. J. Science*, 1914, B, ix, 307 (reviewed in *Ztschr. f. wiss. Mikr.* 1915, xxxii, 82).

All three instruments have been used for most delicate operations. Theoretically, the one which uses the least amount of frictional surfaces in its fine movements should withstand the longest usage without impair-

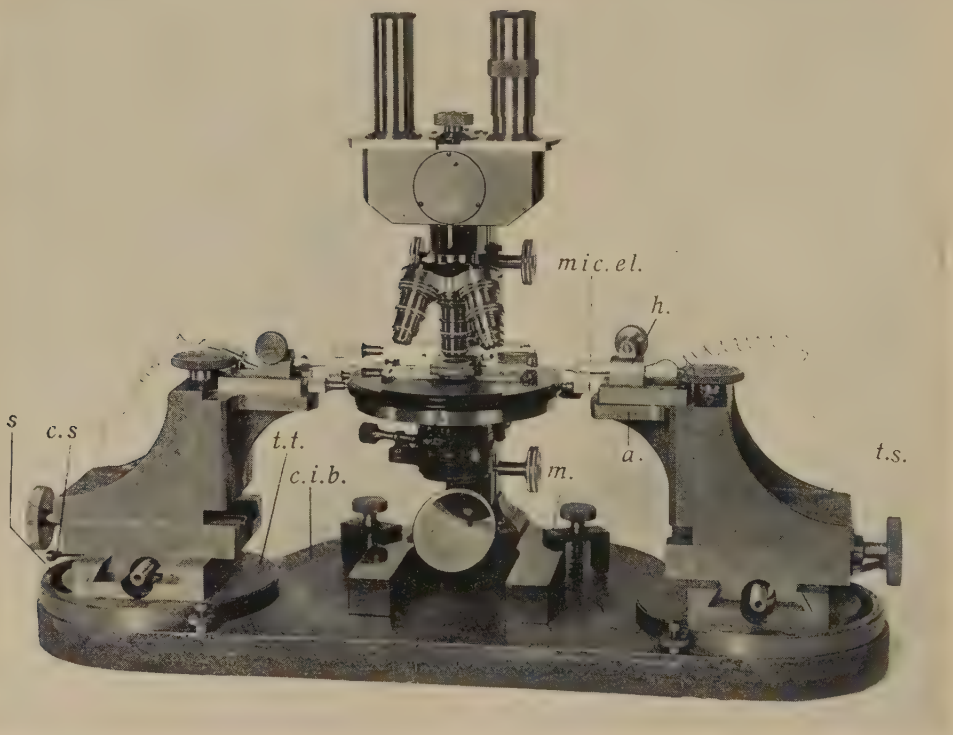


FIG. 4. The Taylor double micromanipulator equipped with microelectrodes. The moist chamber on the stage of the microscope is open at two ends to permit entry of the microneedles; a, arm supporting holder for operative instrument; c.i.b., cast iron base; c.s., clampscrew for turntable; h, holder for operative instrument; m, clamp for microscope; mic. el., microelectrode; s, slot in turntable, t.s., thumbscrew of manipulator; t.t. turntable. (From U. of Calif. Publ. in Zool. 1925, xxvi, 443.)

ment. In Chambers' instrument the frictional surfaces are at a minimum. The advantage which the other two possess is that they can be placed at any distance from the microscope stage without affecting the relative directions of the horizontal and vertical movements.

For most purposes there is no necessity for an absolutely rigid table upon which the instruments are to rest. In all three, the micromanipulator and the microscope are rigidly mounted on a common base so that the entire equipment may vibrate as a unit. A mat of heavy felting beneath the base appreciably minimizes vibrations.

The pillars of the three instruments are adjustable so that they can be made to slide to a considerable distance from the microscope. This facilitates the frequent change of pipettes especially for bacteriological work.* In

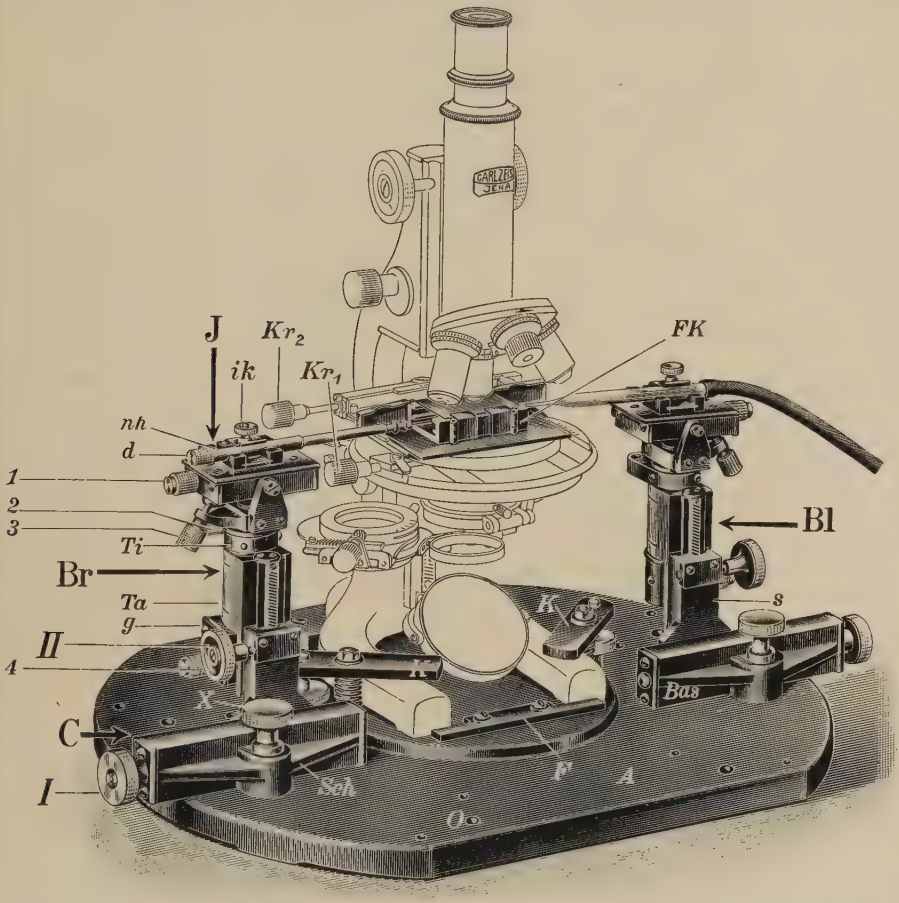


Fig. 5. The Péterfi double micromanipulator. The chamber is mounted similarly to Taylor's. One carrier is equipped with a microneedle, the other with a micropincette the two tips of which can be separated or approximated by means of screws at the base of the pincette. Br, Bl, right and left operating stands; ik, clamp for needle holder (nh); 1, 2, 3, 4, screws for perlateral, sagittal, diagonal and vertical fine movements, d, knob for turning needleholder about its axis. Fk, moist chamber.

Chambers' instrument, (Fig. 7), this adjustment is a removable accessory which may be purchased with the instrument.‖ After the pillar is brought

* Other micromanipulators which have been described in the literature are those of Hecker,† Bishop and Tharaldsen,‡ and Dunn.§

† Hecker, F. *J. Infect. Dis.*, 1916, xix, 306.

‡ Bishop, G. H. and Tharaldsen, C. E. *Am. Nat.*, 1921, liv, 381.

§ Dunn, F. L. *J. Infect. Dis.*, 1927, xl, 383.

‖ Wright, W. H. and McCoy, E. F. *J. Lab. Clin. Med.*, 1927, xii, 3.

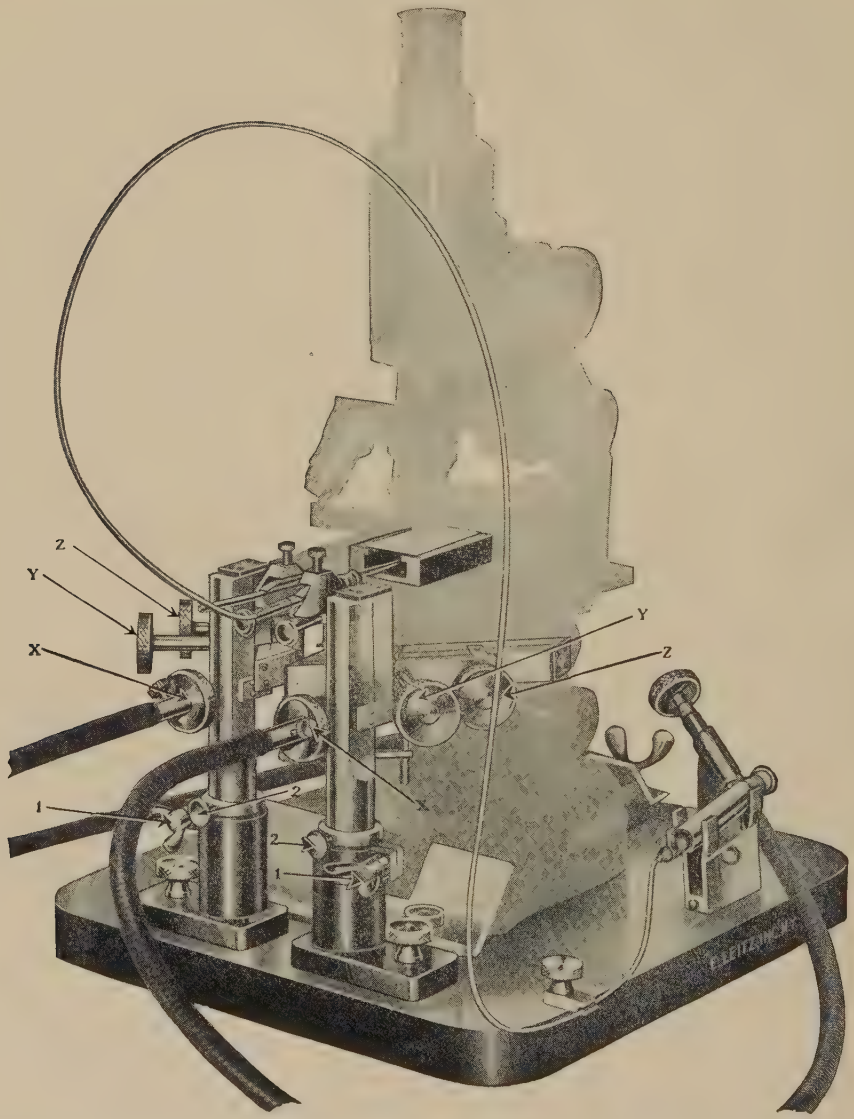


FIG. 6. The Chambers' double micromanipulator mounted so that the microneedles and pipettes project into the moist chamber on the microscope stage from the front only. The vertical movements are furnished with long, curving, flexible shafts which permit their operation from the rear. The syringe, for microinjection, is mounted to the left of the microscope and communicates with the micropipette by means of a wide looped slender brass tube. 1, Clamping screws to hold pillars firmly in place. 2, Clamping screws to arrest pillars at proper height. X, Knurled heads for micrometer movement of needles in perpendicular axis. Y, Z, knurled heads for micrometer movement of needles in horizontal axis.

back to its position close to the microscope it is rigidly clamped. This clamping feature is absent in Péterfi's instrument.

2. Choice of Microscope and Microscope Accessories. The pillars of the Chambers' instrument can be adjusted to the height of any standard microscope. Péterfi's instrument is similarly adaptable but to a lesser degree.

A steady, well controllable mechanical stage is essential in this technique because a great deal of the micro-operative work is done by moving the moist chamber with the mechanical stage. The latter should have a range which allows the moist chamber to be moved far enough so that the free edge of the coverslip roof passes beyond the center of the microscopic field. This enables one to adjust fresh needles and pipettes without fear of striking them against the roof of the chamber.

Probably the best type of microscope stage is a circular rotary stage with a built-in mechanical stage. The rotary feature is frequently of considerable assistance for bringing the open end of the moist chamber into an advantageous position with respect to the shafts of the micro-needles.

The binocular, monobjective microscope is preferable to the monocular type. The equipment of oculars should include a good micrometer ocular and a double demonstration ocular (Fig. 7). Leitz supplies a most serviceable form of the latter with either a high or a low powered ocular.

The selection of high powered objectives must be governed by the fact that considerable depth of focus is desirable for working in hanging drops. For this reason the 3 mm. apochromatic objective is to be recommended. Water immersion lenses should be preferred for a study of fresh tissues and cells suspended in physiological fluids which are largely aqueous. The 3 mm. apochromatic water immersion objective is an excellent one provided that the lens is kept scrupulously grease-free and if due correction is always made for coverglass thickness.

For larger objects, e. g., fresh water amebae, the Leitz No. 5 objective is a most useful one. It can be used with high powered oculars and its free working distance is considerably greater than that of the 4 mm. objective. The 16 mm. objective is always useful and the 32 mm. or, preferably, a Leitz No. 2 objective is convenient for bringing the tips of the microneedles or pipettes into the microscopic field or for finding them when they are accidently shifted from the field.

In order to secure optimum illumination the focus of the substage condenser must reach the roof of the moist chamber which, for ordinary purposes, should not be over 10 mm. high. A triple lens, or, better still, an achromatic, aplanatic condenser can be converted into a condenser having the required focal length by removing the top lens. In some makes the top lens has a screwing attachment for this purpose. In order to take advantage of the full focal length of the condenser the substage must be adjusted to allow the condenser to be brought flush with the upper surfaces of the microscope stage. A simple way to detect the focal length of a condenser is to blow some

smoke on the upper surface of the brightly illuminated condenser whereupon the emerging cone of light is plainly seen. A block of uranium glass placed over the condenser serves the same purpose.

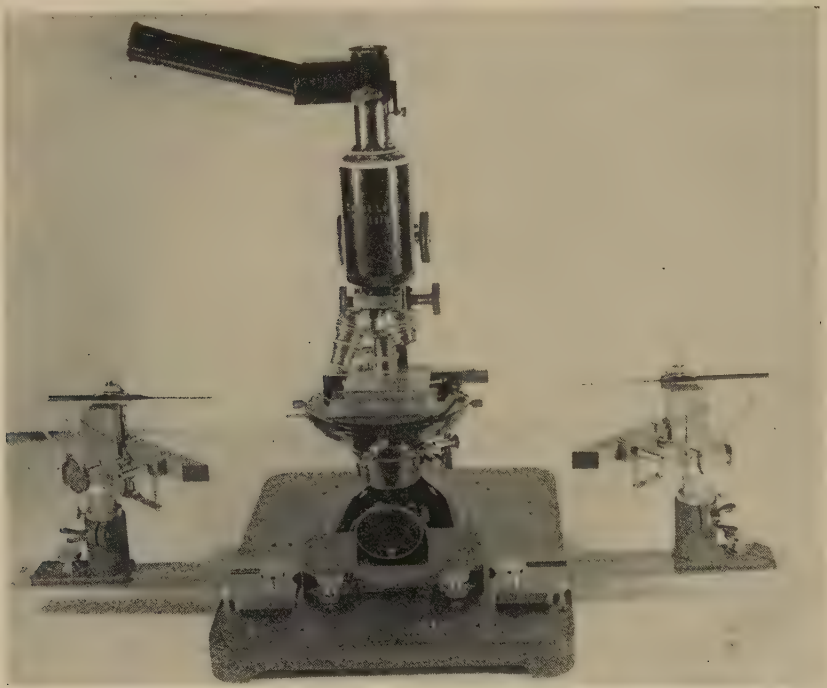


FIG. 7. The Chambers' double micromanipulator mounted so that the microneedles or pipettes project into the moist chamber from two sides. The extension arms permit sliding the instrument away from the microscope for changing the needles. The microscope is equipped with a Leitz double demonstration eye piece.

A serviceable lighting system is a 100 watt electric bulb with a concentrated filament screened by a filter of Gage's daylight, ground glass,* and a spherical flask of water or a bulls-eye lens, placed between the light source and the mirror of the microscope. The position of the flask or bulls-eye can be determined by removing the ground-glass filter and shifting the relative positions of the flask and of the lamp until a sharply defined image of the source of light can be thrown by the plane mirror of the microscope on a distant screen or wall.

The image can be seen either by placing a sheet of white paper on the face of the mirror or by projecting the image on the wall, by means of the mirror. The filter may be placed either between the light source and the flask or between the flask and the substage condenser. The brightest illumination is usually procured by the latter position.

* Gage, S. H. and Kingsbury, B. F. *Anat. Rec.*, 1915–16, x, 527.

Finally emphasis must be laid upon the fact that a study of living cellular phenomena, especially in experimental work in which the elaborated methods of fixing, staining and clearing of tissues have to be discarded, requires a thorough appreciation of the limits and possibilities of critical microscopic vision.*

3. The Moist Chamber and Coverslips. There are two types of moist chambers in general use. One is open at both ends, the other at one end only. The former is used with Taylor's and Péterfi's instrument, the latter with Chambers'.

The chamber designed by Péterfi and supplied by Zeiss is open at both ends which can be closed with loose rubber diaphragms. The microneedles are mounted so that one is on each side of the microscope and their tips are inserted into the chamber through closely fitting collars in the diaphragms.

The type commonly used with Chambers' instrument is open at one end only and is shown in Figure 8. When this chamber is used the two pillars of the instrument are placed so that both of the microneedles or pipettes pass into the chamber from the one end. The base of the chamber is a glass slide having a form which can be tightly gripped in the mechanical stage on the microscope. The walls are strips of glass about two inches long, and of a height which is determined by the free working distance of the available condenser.

This chamber is supplied in two heights, 10 mm. and 14 mm. One end of the chamber is closed with a strip of glass of the same height as the side and backed by another strip a fraction higher. The trough of the chamber should be from three-fourths to seven-eighths of an inch wide. Strips of wet blotting-paper may be placed along the sides of the trough. Frequently it is preferable to have the entire floor of the moist-chamber flooded with water, in which case a thin glass strip is cemented across the floor at the open end. A convenient size of chamber is one which can be roofed over by a 22 × 40 mm. coverslip. The slip is held in place with vaseline. Smaller coverslips may also be used, provided the rest of the chamber is covered with other strips of thin glass.

When several droplets are to be placed on a single coverslip they may be kept separate by rings of paraffin. The rings can be readily stamped on the coverslip in the following way.

A comparatively thin walled glass or, preferably, a metal tube is selected, having an evenly surfaced end with a circumference of the size of the required rings. The end of the tube is heated momentarily in a flame and pressed first on a block of paraffin and then on the coverslip. In this way slender rings of paraffin can be neatly stamped

* Coles, A. C. Critical Microscopy, Lond., 1921.
Beck, C. The Microscope. London, 1924.
Belling, J. The Scientific Use of the Microscope. Carnegie Institute, Cold Spring Harbor. In press.

on the coverslip especially if the slip is kept cold by being laid on a glass plate over a wet towel. The coverslip must be carefully cleaned and grease free in order to ensure the adhesion of the paraffin to the glass.

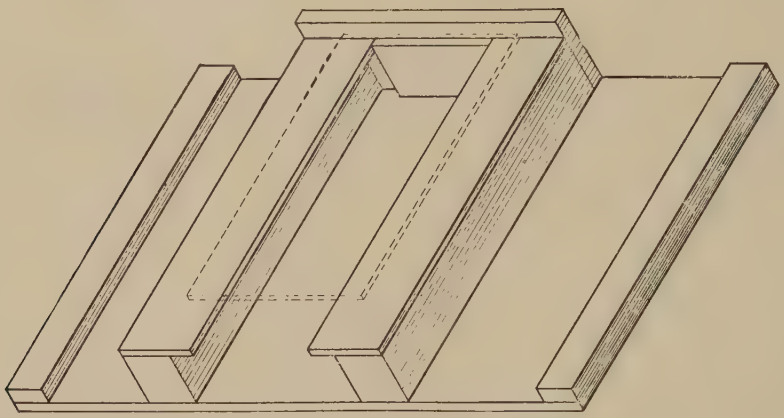


FIG. 8. Glass moist chamber open at one end.

The Needhams* use what they call a "flying coverslip." The chamber is completely covered with a long oblong coverslip to the under surface of which is attached, by a film of water, a small coverslip with a hanging drop containing the tissue or cells to be operated upon. Taylor (p. 43) uses a glass or mica roof perforated with a hole of about 16 mm. to 18 mm. in diameter. The tissue to be operated upon is mounted on a square or round coverslip which is then inverted over the hole and held in place with vaseline.

A circular hole is readily made in a glass slip or coverslip by wetting the glass with emery powder in water and boring the hole with a copper tube which has a circular knife edge on one end. The tube is mounted in a brace or a turning lathe. Another good method for making a hole in a thin coverslip is to fasten the slip securely with paraffin over a hole in a flat metal block. The diameter of the hole should be the same as that required in the glass slip. A tapering pointed metal tool, such as a pointed file, is then plunged through the glass into the hole. The broken edges of the glass, protruding over the hole, are then removed by scraping against the rim with the edge of the file. The perforated coverslip is removed by gently heating the metal block and cleaned in xylol or any other paraffin solvent.

The chamber is purposely made rather long because of the evaporation which occurs at the open end. At a certain distance from the open end is a zone where the water condensation on the roof is at an optimum for maintaining the hanging drop which contains the cells or tissue to be operated upon. In order to prevent excessive condensation it is sometimes advisable to use saline solutions instead of water with which to moisten

* Needham, J. and Needham, D. M. *Proc. Roy. Soc., B,* 1925, xcviii, 259.

the chamber. The open end can be closed by means of a paraffined paper box with its sides cut out to accommodate the shafts of the microneedles. After being set in place the box is filled with vaseline.

A special type of hermetic chamber devised by Barber (p. 43) has been recently developed by the Needhams (p. 50) for dissecting and injecting cells in atmospheres of different gases. The chamber is closed at one end by a trough of mercury through which needles and pipettes on shafts with a U-bend pass into the chamber. The chamber is supplied with two inlets, one to flood the chamber with the gas and the other for a delivery pipette to deposit a hanging drop of a desired solution after the chamber has been filled with the gas. There is also an outlet for the air or gas to pass out. The chamber and the shafts for the micropipettes and needles have been further elaborated with the aid of C. G. Grand in my laboratory and is described in a recent publication.*

A desirable accessory is a moist chamber for preparing and teasing tissues before mounting for micromanipulation.† It consists of a small, bottomless box with a flat glass top and two sides of thin rubber. The box is removably mounted on a plate of glass on which it is held snugly by strips of glass cemented to the plate. A coverslip, with the tissue to be teased, is laid on the glass plate and the box placed over it. The entire chamber is then mounted on the stage of a dissecting microscope and the tissue is teased by means of dissecting needles thrust through the rubber sides of the box.

The cleaning of glass is a most important feature in this type of work in which everything depends not only upon cleanliness but also on the size and shape of the drops suspended from the coverslip and upon the condition of the glass rods and tubing from which the microneedles and pipettes are made.

Glass should be cleaned by boiling thoroughly in soapy water, rinsing and then immersing for hours, in a cleaning mixture of saturated potassium bichromate (one part) and concentrated sulfuric acid (one part). It should then be washed thoroughly in running water, rinsed in distilled water and kept in clean, 95 per cent alcohol. When needed, the glass may be dried with heated air.

Another consideration to be guarded against is the possibility that the alkalinity of ordinary glass and the possible loss or presence of CO_2 in the hanging drop may seriously interfere with the chemical conditions in a microdroplet on a coverslip or in the micropipette.

4. The Microneedles. An automatic machine is being constructed (to be supplied by Leitz) for making the needles and pipettes. It is based on a device of Keith Lucas‡ for making fine glass capillaries. A glass capillary

* Cohen, B., Chambers, R., and Retznikoff, P. *J. Gen. Physiol.*, 1927, xi.

† Chambers, R. *Biol. Bull.*, 1918, xxxiv, 121.

‡ Lucas, Keith. *J. Physiol.*, 1908, xxxvii, Proc. of Soc. xxviii–xxx.

is held rigidly in a vertical or a horizontal position with its free end passing through a coiled loop of platinum. If in a vertical position the free end of the capillary is clamped to a weight; if horizontal, it is clamped to a taut

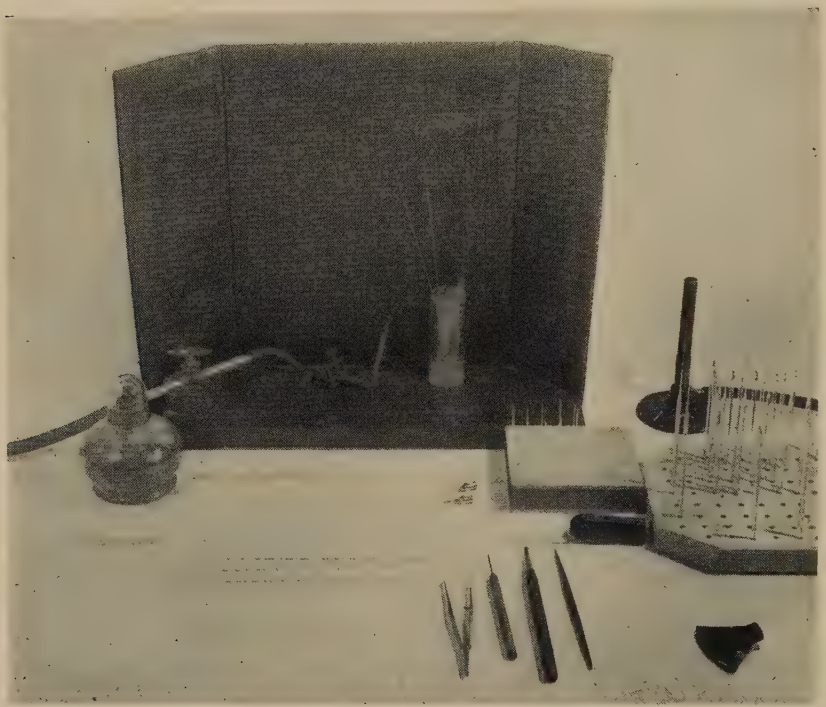


FIG. 9. Equipment for making the microneedles and pipettes. In the glass vial by the gas microburner are several lengths of glass capillaries from which the needles and pipettes are to be made. In the foreground are the implements (flat tipped forceps and steel, dissecting needle), pieces of glass tubing and rods (3–4 mm. in diameter), some of which have been drawn into capillaries. To the right are two wooden stands with holes. One supports a supply of glass rods with capillaries, the ends of which have been drawn into microneedles. On the smaller stand is a supply of micropipettes. Ordinary soft and hard glass capillaries are drawn in a Bunsen flame. Quartz capillaries have to be drawn in an oxyacetylene flame.

spring. The platinum-loop is heated by an electric current and the glass capillary as it begins to melt is immediately drawn out to a point. With little practice, however, it is possible to make microneedles and micropipettes by free hand. The equipment for this purpose is shown in Figure 9 and detailed description of the method follows.

a. Making the Microneedles. (1) *The Glass.* The needles are made from glass-rods or from soft or hard glass tubing (3 to 5 mm.). If tubing is used, the thicker the wall of the tubing, the firmer tends to be the tip of the needle made from it. The technique for making the needles of soft glass is readily mastered. It is somewhat more difficult to make them of hard glass but they tend to be more durable.

(2) *The Capillaries.* The glass rods or tubes must first be drawn in a bunsen flame into capillaries at least 7 cm. long and with an outside diameter of about 0.5 mm. to 1.0 mm. For quartz glass a hotter flame is required.

If the capillary is drawn from a 3 to 5 mm. rod, a portion of the rod may be left as a shank for mounting in the carrier of the micromanipulator (cf. Fig. 11, a, b). Usually, however, the capillaries are prepared without shanks (cf. Fig. 11, b). After the microtips have been made according to (4) the capillaries are stored in a perforated block of wood (cf. Fig. 9). When a capillary, lacking a shank is to be used, its shaft is cut down to the proper length with a carborundum or diamond pencil, and is fastened, (cf. Fig. 13), into the perforated end of a special glass or metal holder which fits the carrier of the micromanipulator. For this purpose it is advisable to use a gauge, in the form of a short length of glass tubing with a bore equal to that of the special holder. The capillary lengths from which the needles are to be made can then be selected from those which fit the gauge.

(3) *The Microburner.* The microburner, (Fig. 9), is made from a piece of hard glass tubing bent almost at a right angle and with the upturned end closed except for almost the smallest possible aperture that will retain a flame. This can be done by heat-softening one end of the tube and pinching it with a pair of forceps or by allowing the end to melt down to a fine aperture. When set in place the tip of the burner should stand at a height somewhat less than the width of the operator's palm measured across the base of his fingers, (cf. Fig. 10). The size of the flame is regulated by one or two screw pinchcocks on the rubber tube connecting the burner with the gas jet. A steel hypodermic needle mounted in a rubber tube will also serve as a microburner.

(4) *The Microtips.* (*a*) The Flame. Lower the flame of the microburner to almost the smallest flame that will remain lighted. The microburner should be protected from draughts of air and kept in semi-darkness or against a mahogany red background so that the flame may show up to best advantage.

(*b*) Length of Shaft. The shaft when mounted, should be about 5–6 cm. long. This length depends upon the length of the moist chamber and the distance of the field of the microscope from the carrier of the micromanipulator. If the capillary shaft is too short its holder will extend into the moist chamber and so impede the range of movement. On the other hand, if the shaft is too long the carrier will not hold it.

(*c*) Drawing the Microtip. To make the microtip on a shaft provided with a shank hold the shank in the left hand and grasp the capillary either with forceps or with the thumb and forefinger of the right hand. If the microtip is to be made on a free capillary unattached to a shank hold it as in Figure 10. In this case no special precaution is required regarding length as the shaft is cut later to the proper size. A convenient length at this stage is 5 cm. to 6 cm.

While pulling gently and steadily with the right hand bring the capillary just over the microflame, (Fig. 10, A, B).

As the glass is softened by the heat, lift it slowly from the flame and pull slightly more than at first, but not too strongly. The hands should rest on the table during the procedure and the pulling and lifting done by turning them slightly outward, (Fig. 10, C). The capillary will separate with a slight tug—a feeling much like that experienced when a taut thread, held in the fingers, is parted in a small flame. If the needle is properly made, as in Figure 11, c, d, it will taper to a fine, rigid tip. Everything depends upon the amount of heat used and the timing of the pull, and these vary slightly with the height of the flame and the diameter of the capillary. With a little experience, one can usually tell when a proper tip is made by the peculiar feeling described above. If too little heat is used and the pull made too suddenly, the capillary may part with a snap and the tip will be found broken off short or if the capillary is hollow, it may form a tapering, perforated tip which is unserviceable for any delicate work. If too much heat is used, the capillary will draw out into a long flexible, unserviceable hair, (Fig. 11, e).

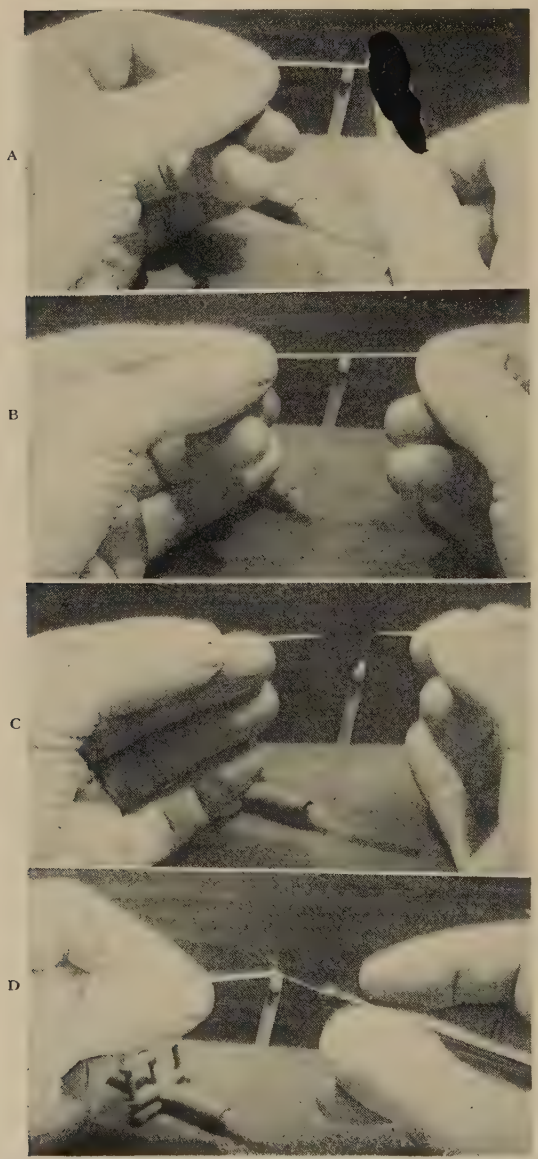


FIG. 10. Four photographs showing various stages in making the microneedles and pipettes from a glass capillary. A, short capillary being held by forceps over microburner preparatory to drawing out a needle point; B, similar procedure with a long capillary held by both hands; C, the tug which separates the heated capillary in the formation of the needle tips; D, bending the shaft of the microneedle or pipette in the microflame.

The type of needle tip and its serviceability is determined upon examination through a low power of the microscope.

(*d*) Types of Microtips. A successfully made microneedle (Fig. 11, b) on the end of

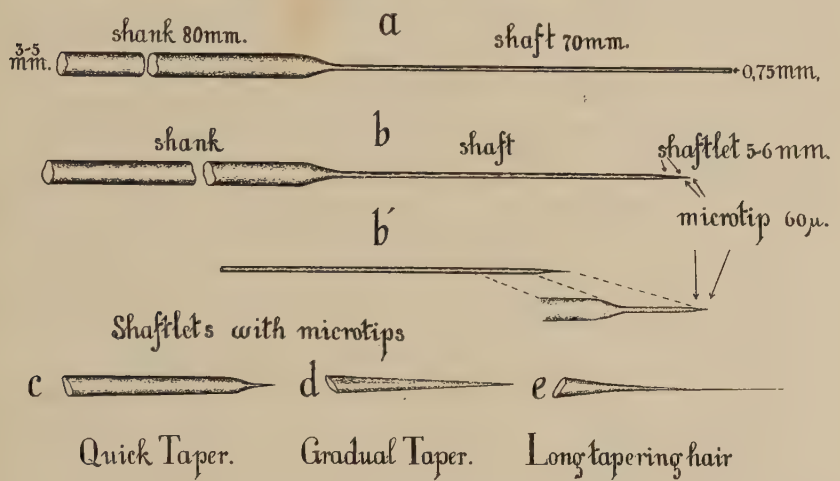


FIG. 11. Making of microneedles; a, glass shank (3–5 mm. in diameter) drawn out into a capillary about 0.75 mm. in diameter and 70 mm. long; b and b′, capillary shaft with and without a shank. The end of the shaft is drawn out, in a microflame, into a shaftlet, 5–6 mm. long and a microtip about 60 micra long; c, shaftlet tapered quickly into an elongated microtip (quick taper); d, shaftlet with a gradually tapering microtip (gradual taper); e, unserviceable, long, tapering hair.

a capillary shaft consists of a shaftlet and a microtip. The needles are of various types according to the form and size of the shaftlet and of the tip. It is usually impossible to tell beforehand which of these types will be made when drawing them out over the microflame.

TABLE I

Taper	Size	Diameter of Shaftlet	Diameter of Microtip at Base	Length of Microtip	Diameter of Opened Tip
Quick	Coarse	35 μ	8 μ	60 μ	2–5 μ
	Medium	10–15 μ	3 μ	35 μ	½–2 μ
	Slender	6–10 μ	2 μ	10–20 μ	½–1 μ
		Diameter of Shaftlet 30μ from Microtip			
Gradual	Coarse	5 μ			
	Medium	3 μ			
	Slender	2 μ			

Table giving actual sizes of the two serviceable types of microneedles, the quick taper (cf. Fig. 11, c) and the gradual taper (cf. Fig. 11, d), classified as coarse, medium and slender.

The serviceable needles are classified into two general types, one, the quick taper, Fig. 11, c, in which the diameter of the shaftlet decreases more or less rapidly into a slender, elongated tip and the other, the gradual taper (Fig. 11, d) in which the shaftlet

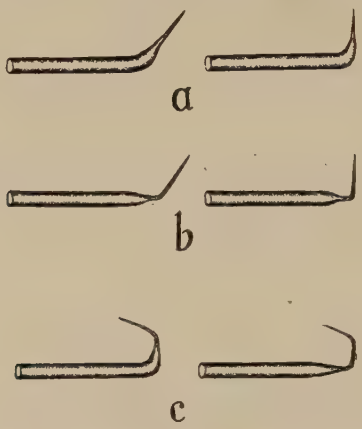


FIG. 12. Bending of shafts of microneedles. a, Rigid needles with bend on the shaft; b, yielding needles with bend on the shaftlet; c, rigid and yielding needles with shaftlets bent on themselves.

tapers gradually to the tip. Both types may be further classified as coarse, medium and slender. Some actual measurements from typical needles are given in Table I.

With experience the operator will soon learn to appreciate the type of needle best adapted to his special requirements.

b. Bending the Shaft. After a suitable microtip is made the needle is bent at an angle over the microflame (Fig. 10, D). The bend should be anywhere between a right and an obtuse angle of about 135° or more. In Figure 12 several different types of bends are shown. A rigid needle results if the bend is on the shaft just behind the slender shaftlet (Fig. 12, a). This type of needle, especially if the bend approaches a right angle, is in danger of being easily broken when the tip meets the coverslip. A more resilient and yielding type results when the bend is made on the shaftlet (Fig. 12, b) especially when the angle of the bend is obtuse. A type of needle (Fig. 12, c) in which the shaftlet is bent upon itself, is well adapted for cutting or moving a cell about.

The method of bending varies with the difference in the length of the shaftlet. If the shaftlet is long enough to be included in the knee of the bend, the heat of the microflame is often sufficient in itself to produce the bend. A metal needle or a broad tipped forceps serves not only to protect the tip from the flame during the bending process but also to conduct away the heat which may otherwise completely melt the shaftlet. If the shaftlet is short the knee of the bend is made on the capillary shaft beyond the shaftlet and this requires pushing with the steel needle or with the forceps. The

height of the portion of the needle beyond the bend should not be over 4 mm. or 5 mm. for use in a moist chamber 10 mm. high.

The grasping surfaces of the forceps used in drawing out the needles

FIG. 13.

should be more or less parallel. In order that the surfaces may grip without breaking the glass it is advisable to coat them with a thin layer of Canada balsam or varnish.

Fine points of shaftlets of serviceable length are not made readily from capillaries of a diameter much greater than 0.5. to 0.8 mm. Therefore, if a comparatively stiff shaft is required, start with a thick capillary and draw out the tip into a thinner capillary. This thinner capillary may then be used for making the microneedle.

c. Mounting the Needles. The needles are fastened into their shanks with cement,* or by a set screw and rubber washer recently devised in my laboratory by H. Frank.† The shanks are now mounted in the carrier of the micromanipulator so that the needles project into the moist chamber on the microscope stage (Fig. 14, cf. Fig. 16).

First, one needle is coarsely adjusted by bringing it into the cone of light coming from the substage condenser. Secondly, the tip of the needle is centered, first with the 32 mm. and afterwards with the 16 mm. objective. A narrow strip of coverslip is then laid across the moist chamber outside the microscopic field and beyond the needle. While the tip of the needle is being kept under observation through the 16 mm. objective the moist chamber is moved with the mechanical stage, until the edge of the coverslip comes into view beyond the tip. The tip of the needle should now be raised or lowered with the coarse adjustments of the micromanipulator to a level from which, by means of the fine adjustments of the micromanipulator, it can be raised easily to the undersurface

*The cementing of glass to glass or glass to metal is a feature of considerable importance in this technique. The cement should be of chemically inert material which hardens immediately and which can be readily softened and removed when desired. The de Khotinsky cement (medium or hard quality) obtainable in this country is well suited for this purpose. Péterfi uses colophonium wax (colophonium, 3 parts, and beeswax, 5 parts). Another cement which may serve is a mixture of 8 parts commercial resin and 2 parts anhydrous lanolin. The proportions of the constituents may be varied to change the consistency. Shellac is also good.

†This device (Fig. 13), supplied by Leitz, may be substitued for the de Khotinsky seal described in the text (p. 64). It consists of a hollow metal shank 3 inches in length and of a thickness to fit the needle carrier of the Chambers apparatus. The shank is provided on one end with a tightening nut enclosing a perforated rubber washer. The shaft of the microneedle or micropipette is inserted through the rubber washer in the nut and the nut is then tightened by simply turning it with thumb and finger. The other end of the shank is threaded so that if it is to be used for microinjection it can be joined with the brass or copper tubing. (cf. Fig. 15, 3.)

of the coverslip and as easily lowered so as to be free from the deepest hanging drop which is to be used. The details of this procedure vary somewhat with the type of instrument and the moist chamber.

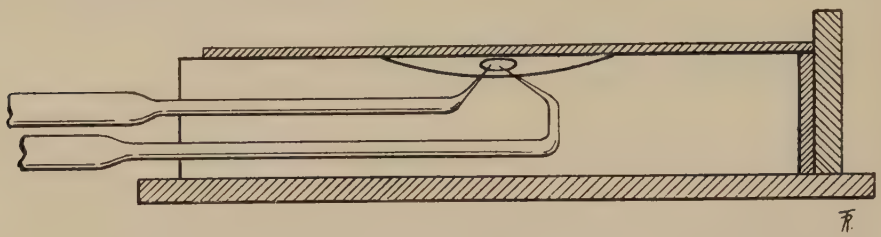


FIG. 14. Needles mounted in a moist chamber. Side view of moist chamber to show two microneedles with their tips in a hanging drop suspended from a coverslip which roofs the moist chamber.

When once the first needle is properly adjusted it is a simple matter to repeat the procedure for the second by using the position of the first one as a guide.

A good needle, when once proven to be resilient and strong, can be used repeatedly for a long time.

When two microneedles are to be used, one may be bent backward and the other forward (Fig. 14). The tip should not point directly back but should slant off to one side, so that the shaft of the capillary will not be in the way of the light. For a cutting needle the angle at which the shaft is bent back on itself can be ascertained only by experience, for, if it is too near the horizontal, the tip, as it is raised against the coverslip, will tend to bend down instead of cutting up into the cell or tissue to be operated upon. If the angle is too far from the horizontal, the cell will slip away from the needle.

d. Other Methods for Obtaining Needle Tips. Another good method for making the needle-points is that of Chabry (p. 40). The tip of a glass capillary is brought into contact with a heated mass of glass (or any incandescent body to which glass will adhere) and suddenly drawn away. For an incandescent body Chabry used the blade of a platinum knife. The capillary is held in a groove on a stand a few centimeters from the platinum blade. The platinum blade is then heated to a dull glow, and the capillary slid in the groove until the tip touches the glowing metal when it is instantly slid back. The sliding of the capillary in a stationary groove insures a straight taper.

Péterfi (p. 43) has an ingenious micro-cautery device for drawing glass points in the field of the microscope.

Needlepoints may also be made by grinding one end of a fine wire. To produce chemical effects in a cell, McClendon* used a copper-wire ground to a point and further sharpened by erosion in acid. The chemical injury produced by metal needles limit their use for the dissection of living cells.

* McClendon, J. F. *J. Exp. Zool.* 1909, vi, 265.

A number of ingenious minor accessories such as micropincettes, scales of butterfly wings cemented to the tips of microneedles, etc., have been described by Péterfi (p. 43). Chabry also suggested the use of insect mouthparts, annelidan bristles and the spicules of sponges for the tips of micro-dissection needles. Barber (p. 43) suggested fine-pointed needle-crystals, and the sharp, stiff hairs on the body of a house fly. A pipette with a fairly large opening is made and the hair or crystal is drawn partially into it. The fine point projecting from the tip of the pipette is then used as a probe or dissecting needle.

5. Cellular Microinjection Apparatus and Technique. The microinjection apparatus is used with a micromanipulator for the purpose of injecting fluids or granules into the cytoplasm or nucleus of living cells. It can also be used to withdraw materials from cells. The diameter of the bore of the micropipettes may be anywhere from less than half a micron to more, according to the required type of injection.

The ability to microinject depends not only on the fineness of the micropipettes but also on the accurate control of the pressure.

In the methods described by Barber (p. 43) and by Péterfi (p. 43) the driving force depends upon the effect of heat and cold on the expansion of mercury on the one hand and air on the other. For finer work it is necessary to have a more rigid control over the injection than is afforded by these means.

More serviceable methods are those of Chambers (p. 43) and Taylor (p. 43).

a. Chambers' Injection Apparatus. This apparatus (Fig. 15) supplied by Leitz, consists of a glass Luer syringe (1 to 2 c.c.), preferably with a metal nozzle, 1; a slender, soft, brass capillary tube, 2; (less than 2 mm. outside diameter and about 1 meter long) which connects the syringe with a nozzled, metal shank, 3, (about 4 mm. to 5 mm. in diameter and about 6 cm. long); and a metal adapter, 4, (hypodermic needle with its needle cut off about 2 cm. from its base) which accurately fits the nozzle of the metal shank. The plunger of the syringe is operated by hand or by a turn-screw or a lever.

The apparatus is assembled as follows:

The syringe and the lower end of the flexible brass tube, which connects with it, are clamped in place close to the base of the microscope. The brass tube, running from the base-clamp, Figure 15, 5, is curved and bent into a large loop until the shank, 3, lies, with no constraint, in the carrier, 8, of the micromanipulator where it is to be clamped with a screw. Finally, the adapter, 4, without the micropipette, 7, is mounted on the nozzle of the shank, 3. To prevent leakage it is advisable to smear the nozzle with a film of beeswax warmed to the melting point just before mounting the adapter. The syringe, the flexible tube and the shank with the mounted adapter are then completely filled with water (preferably deaerated) and the plunger pushed at least half way into the syringe. The entire system is thus full of water except for the micropipette which is to be mounted in the adapter and which contains air.

The Needhams (p. 50) have introduced a useful modification whereby the syringe can be filled by a continuous supply of water. Between the syringe and the pipette there are inserted two capillary, three-way stop-

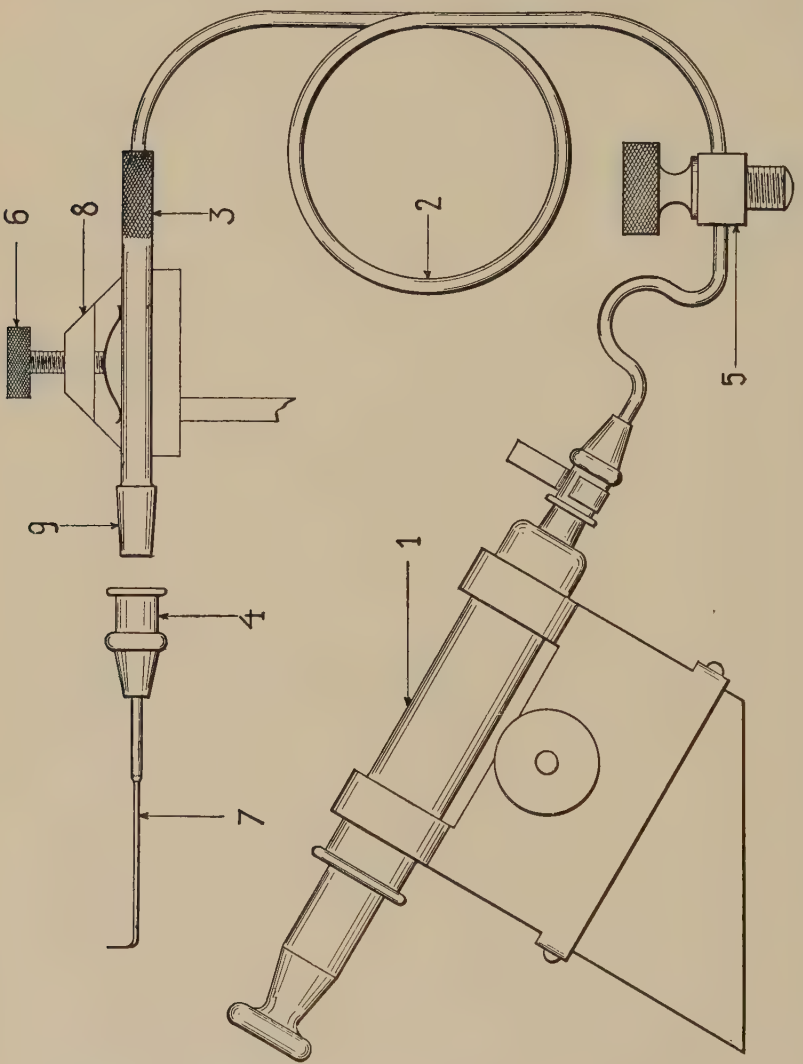


FIG. 15. Chambers' microinjection apparatus. 1. "Luer" syringe of 1 or 2 c.c. capacity mounted in a clamp. 2. Slender, coiled brass tube, one end of which connects with the syringe and the other end with 3, metal shank which is clamped in 8, the needle carrier by means of 6, a clamping screw. 4. An adapter into which 7, a glass micropipette is fastened. 5. Base clamp to fasten brass tube to base.

cocks, one leading to a reservoir of boiled water, and the other opening to the exterior. By opening the former the syringe can be filled by raising the plunger; by opening the latter the amount of liquid in the system can be diminished.

(1) *Micropipettes.* The micropipettes are made from hollow, preferably thin-walled, hard glass capillaries drawn to needlepoints exactly as described

on pages 52 to 59, (cf. Fig. 11). An essential precaution in the bending of the shafts of such needles is to maintain a wide lumen at the bend. To detect this the knee of the bend should always be inspected with a low powered microscope. The conversion of the hollow needles into micropipettes is done, just before using, by breaking off their tips (p. 53).

Hard or alkali-free glass tubing is used for two reasons; first, the brittleness of hard glass enables one to break off the tip with greater readiness, and second, hard glass can be more readily cleaned and rendered alkali-free. The use of thin-walled tubing is to insure having the largest bore possible in the slender shaftlet of the pipette. Sometimes it is more convenient to have pipettes with stouter walls. Such pipettes are less readily broken but, owing to the smaller sized lumen, run the risk of quickly clogging. The best pipette for general use is of the quick tapering type, (Fig. 11, A and Table I). Its aperture, when first made, may be less than half a micron in diameter and, in spite of this, fluids will flow through it with the utmost ease. The comparatively broad shaftlet close to the tip serves to supply an amount of pressure which may enable one frequently to force open a clogged microtip.

(2) *Mounting the Micropipettes.* The usual procedure (Fig. 16) is to have the adapter left permanently on its shank. The shank is temporarily clamped in a holder on the table to one side of the microscope (Fig. 16, C). The entire apparatus is filled with water from the syringe. The micropipette is then fastened into the adapter according to the instructions which follow. When a used micropipette on the adapter is to be discarded it is removed by heating the cement at the joint and is replaced by a fresh micropipette. However, after the technique of sealing the micropipette in its adapter has been fully mastered it is possible to fit several adapters, each with its own micropipette, to be exchanged whenever desired (Fig. 16, D). The process of sealing the joint with cement is a nuisance and should be replaced if possible by the use of the metal shank described in footnote on page 57.

The following precautions adapted from Taylor (p. 43) should be observed in mounting the micropipette into the adapter.

(*a*) See that the pipette shaft fits the bore of the adapter fairly closely. With a diamond or carborundum pencil cut it into a length of 2 inches, more or less, depending upon the distance of the carrier of the micromanipulator from the center of the microscopic field.

(*b*) Draw out a stick of heat-softened de Khotinsky cement (p. 57), about half the diameter of an ordinary lead pencil and 2 or 3 inches long.

(*c*) After carefully melting (without boiling) the tapered end of the stick draw a small ring of the melted and hot cement around the pipette shaft about 2 to 4 mm. from the end which is to be sealed in the adapter, and allow the ring to harden. This is shown in Figure 16, A.

(*d*) Heat the tip of the adapter just enough to melt the ring of cement around the shaft when the latter is introduced into the adapter (Fig. 16, B).

In Chambers' apparatus a slight pull on the plunger of the syringe at the moment of the above procedure often aids in sealing the joint. If a leak persists more cement may be applied around the joint with a hot needle. Since the water in the apparatus extends

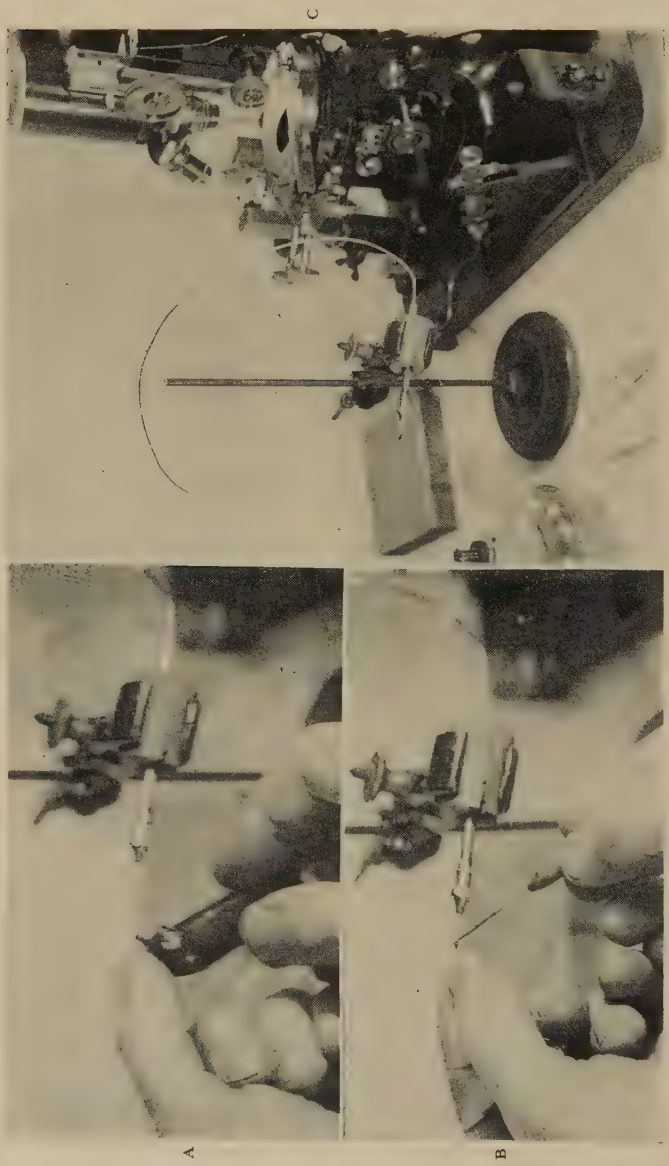
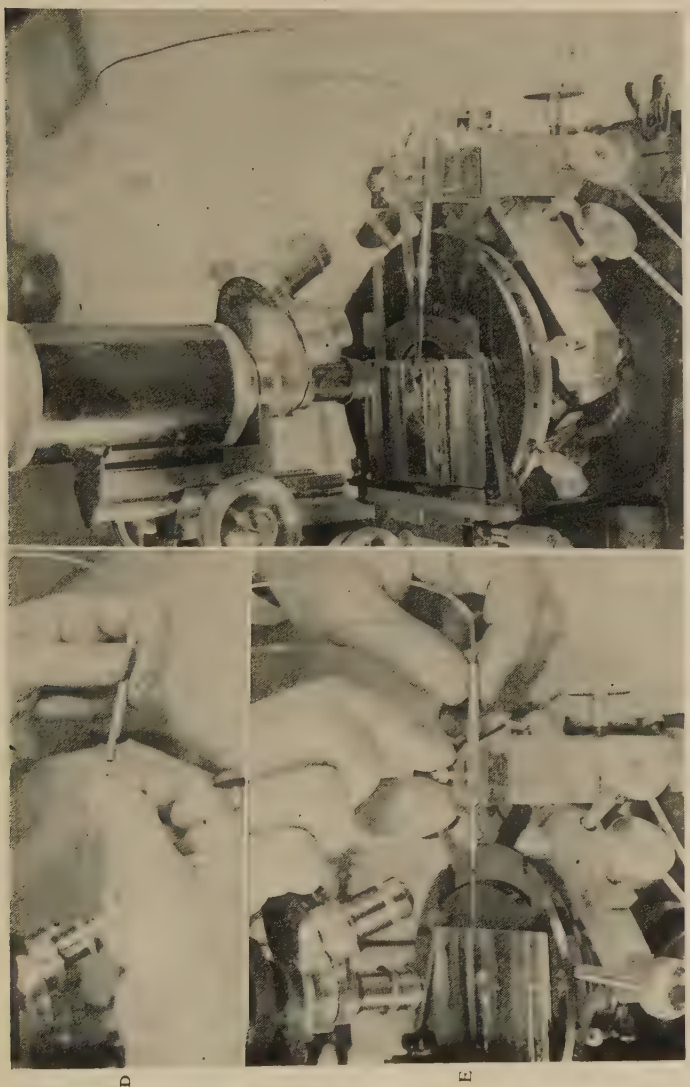


FIG. 16. Photographs showing the method of mounting the micropipette in Chambers' injection apparatus. A, the base of the shaft of the pipette being wrapped with a layer of melted de Khotinsky cement; B, the shaft of the pipette inserted into the metal adapter is being sealed by melting the cement with a heated dissecting needle; C, the micropipette sealed in the adapter and ready to be mounted in the micromanipulator. (Before the pipette was sealed in the adapter the syringe, at the base of the microscope, and the brass tube, leading to the adapter, should be completely filled with water.) D, mounting the micropipette with its adapter on the nozzle of the metal

into the adapter the patency of the glass capillary at the sealed joint can be tested by pressing on the plunger of the syringe. If the capillary is open, water will be driven into the lumen of the capillary beyond the seal and will be seen. When the plunger is released the water will move back if the tip of the micropipette is still closed as it should be.

(3) *Opening the Microtip.* In order to complete the set up for an injection experiment the shank, on which has been mounted its adapter with a micropipette, is clamped into the carrier of the micromanipulator (Fig. 16 D).



shank. This method is sometimes resorted to when the pipettes are sealed into several adapters for rapid exchange. E, the shaft of the micropipette being mounted in the carrier of the micromanipulator. F, one micropipette and one microneedle mounted and ready for use. (During this preliminary adjustment the moist chamber has been moved back so that the tips of the needle and pipette are outside the chamber although they lie in the field of the microscope.)

The pipette, which is still closed at its tip, is then pushed into the moist chamber on the stage of the microscope and adjusted according to the directions on page 57. Its tip is now brought into the hanging drop of the fluid to be injected, and is converted into an open micropipette by raising the tip very cautiously and breaking it against the surface of the coverslip either in or out of the drop. Three precautionary measures are necessary

during this procedure, first, the tip should be broken while under observation through the objective which is to be used for the injection. This ensures against breaking off too much of the tip. The second precaution is to use the tip of the microneedle, mounted in the accompanying micromanipulator to orient oneself regarding the proximity of the undersurface of the coverslip. In other words, the needle should serve as an advance guard of the pipette in order to minimize the risk of damaging the latter. Finally, it is advisable to keep the plunger of the syringe under pressure while the tip of the micropipette is being broken. This lessens the risk of clogging the pipette at the very start.

The injection fluid is now drawn into the pipette by pulling the plunger of the syringe.

Operating the plunger of the syringe for expelling required quantities of fluid from the micropipette requires a little practice. An essential precaution for controlling the delivery of minute quantities is to have a microtip of the rapidly tapering type (p. 55, Fig. 11, c). Pushing and pulling of the plunger of the Luer syringe should be performed by gently rotating the plunger between the left thumb and forefinger with the wrist firmly steadied on the table.

From the preceding description it is evident that the injection fluid in the tip of the micropipette is separated from the water in the rest of the injection apparatus by a column of air in the shaft of the pipette.

The hanging drop should be as free as possible from suspended particles which may clog the pipette. Particles tend to sink and will in time accumulate in the lower surface of the deepest region of the drop. Therefore, especially with a very fine pipette, always fill the pipette while its tip is far up in the drop, and when removing it from the drop, be sure not to lower it through the deepest region where the particles tend to accumulate.

b. Taylor's Injection Apparatus (p. 65). Injection with this apparatus* (Fig. 17) is performed by mechanical pressure on a column of mercury with screws which press against thick rubber diaphragms. For filling the apparatus with mercury the air is evacuated by means of a vacuum pump and all glass parts must be scrupulously clean. When ready for injection the mercury should extend to the tip of the micropipette and fluids are drawn in by reversing the screw.

The micropipette is cemented to the shank of the apparatus essentially as described on page 57. However, greater precautions have to be taken because all air bubbles must be rigidly excluded. This is a more essential feature in a system filled with mercury than in one filled with water. Moreover, the de Khotinsky cement, if overheated, liberates a gas which, escaping into the shank, seriously affects the surface tension of

*Procurable from V. Arntzen, mechanician in the Department of Civil Engineering, University of California.

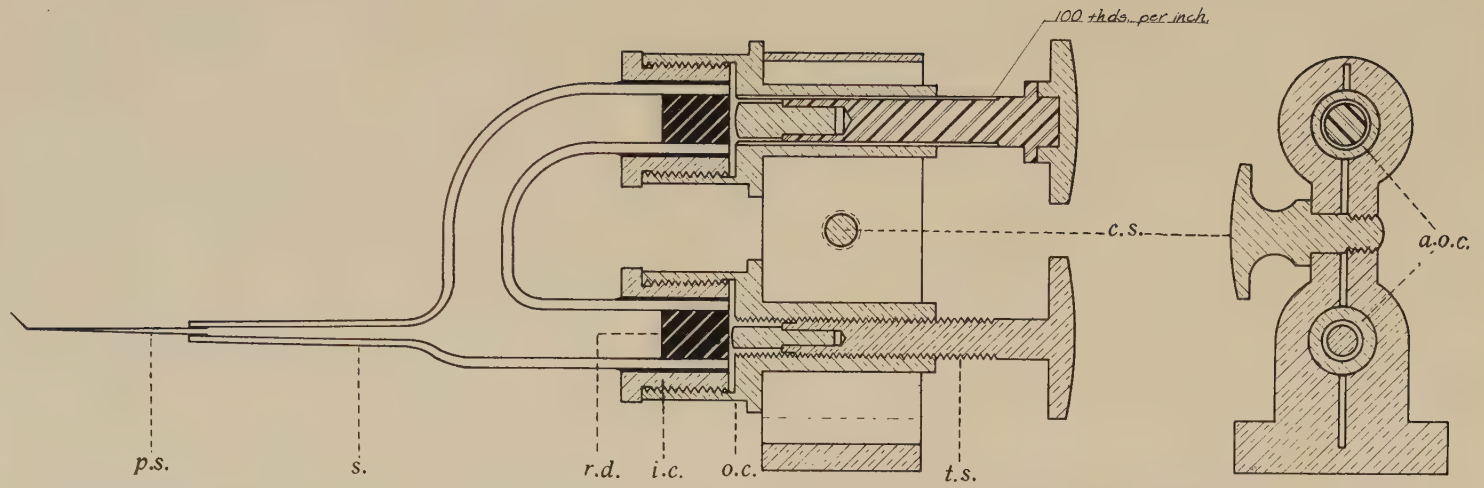


FIG. 17. Taylor's microinjection apparatus furnished with a coarse and fine screw; c.s., clamp screws; i.c., metal inner cap; o.c., metal outer cap; p.s., micropipette shaft; s., glass shank into which micropipette shaft is inserted and cemented, and which is filled with mercury; r.d., rubber plug; t.s., thumb screw. The weight of Taylor's micromanipulator permits this injection apparatus to be mounted directly on the carrier. With Chambers' micromanipulator it has to be mounted on the base beside the foot of the microscope with a looped, flexible metal tube connecting the shaft, s., with the micropipette in the carrier of the micromanipulator. (From U. of Calif. Publ. in Zool., 1925, xxvi, 443.)

the mercury as evidenced later by the breaking of the column of mercury in the pipette tip.

The pipette shaft having been sealed in place, the mercury is then forced to the tip of the pipette by turning both thumb-screws against the rubber diaphragms. The apparatus is now ready for use.

6. Microinjection Apparatus for Renal Corpuscles, Blood Capillaries, Etc. Wearn and Richards* used a Barber Pipette Holder (p. 43) to hold a sharp-pointed, quartz capillary pipette (point with 10 μ to 20 μ inner diameter) for withdrawing fluid from the renal capsule of a frog's kidney. The operation is performed in the field of a binocular dissecting microscope. The pipette is connected by means of rubber tubing and a 3-way stopcock with a bulb and the whole apparatus, including the quartz tip, is filled with mercury from the bulb. After inserting the tip of the pipette into the renal capsule the bulb is lowered to create negative pressure sufficient to draw the glomerular fluid into the pipette. For the technique of preparing the frog, arranging the illumination of the kidney and of analyzing the withdrawn fluid the reader is referred to the original article.

White and Schmitt† used a similar method for the kidney tubules of Necturus. For the pipette holder they devised a metal pipette holder of comparatively simple construction which permits manipulations sufficiently accurate for their purposes. A full description of it is given in their publication.

Landis‡ has modified Chambers' microinjection apparatus to include a manometer for recording the capillary blood pressure and to permit injections under known pressures, (Fig. 18). When working with the mesentery of a frog a loop of intestine is pulled out and laid loosely over a glass slide. The preparation is kept moist by a constant slow drip of normal saline solution. The following description is quoted from Landis' paper:

> The microscopic implements consist of a blunt rod and the micropipette. The rod is made by fusing a small ball, 20 to 50 micra in diameter, on the end of a glass microneedle. This is a convenient implement to stop flow in a capillary temporarily or permanently by pressure, or to hold the mesentery firm during the introduction of the pipette into the more resistant larger vessels. The micropipettes are made of Pyrex capillary tubing with the small gas flame or microburner described by Chambers (p. 53). The tip is broken off against the edge of a glass slide until the diameter of the orifice is from $\frac{1}{6}$ to $\frac{1}{3}$ the length of a frog's red corpuscle, i. e., from 4 to 8 μ.
>
> The movements of each implement are governed by a Chambers micromanipulator. It has been necessary to make some changes in the arrangement of the separate parts. The pipette and rod both come to the microscopic field from the right side of the stage, and the pipette is directed at a gentle slope downward instead of sharply upward as in a hanging drop preparation.
>
> The other end of the micropipette is connected with a device for the application and measurement of pressure (Fig. 18). This consists essentially of a reservoir; R, a

* Wearn, J. T. and Richards, A. N. *Am. J. Physiol.*, 1924–25, lxxi, 209.

† White, H. L. and Schmitt, F. O. *Am. J. Physiol.*, 1926, lxxvi, 483.

‡ Landis, E. M. *Am. J. Physiol.*, 1925–26, lxxv, 548.

syringe, S, for exerting large positive or negative pressures; and a column of water, W, by means of which a measured amount of pressure up to 30 cm. of water may be applied to the contents of the micropipette. A dye solution may be drawn into the tip of the

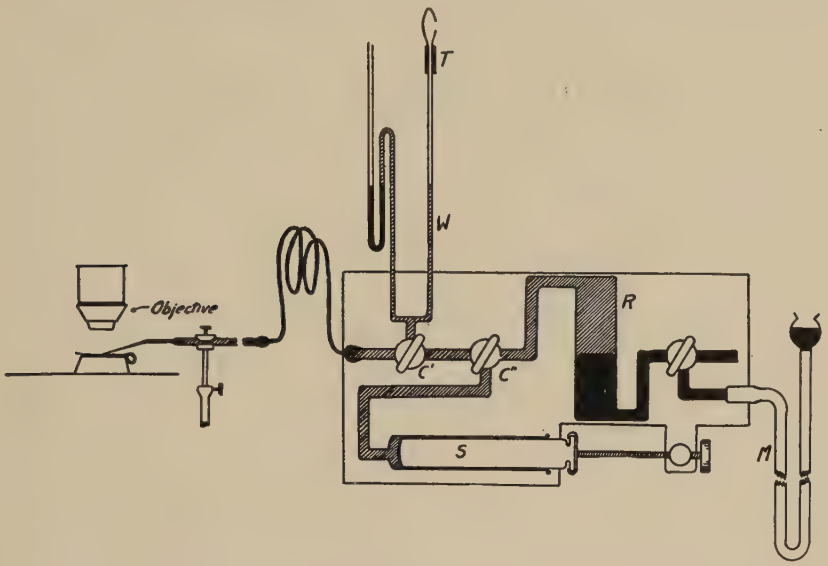


FIG. 18. Diagram of Landis' apparatus for measuring capillary blood pressure. For description see text.

micropipette by a column of mercury, M, or by carefully withdrawing the plunger of the syringe when the two stopcocks, C^{1}, C^{11}, are properly arranged. For accuracy of control the plunger of the syringe is moved by a screw adjustment.

After the pipette has been introduced into the capillary the stopcocks are so turned that the pressure is applied to the dye in the pipette by the column of water, W, the height of which can be changed rapidly by the movement of the syringe plunger. To correct for capillarity in the upright tube the instrument has been calibrated by balancing the water column against known pressures. For measuring the higher pressures found in arteries a mercury column is more convenient. This is connected through a U-tube with the water manometer. If the opening at the tip of the tube, W, is open the whole system functions as a column of water, but if it is closed by a ringlet of rubber tubing, T, the pressure applied is measured by the mercury manometer providing correction is made for the column of water between the surface of the mercury and the tip of the pipette. This arrangement allows a more rapid succession of pressure determinations in arterioles and arteries than would otherwise be possible.

Florey* describes a technique for working with vessels in the mesentery of small mammals.

7. Microelectrodes and Micromagnets. These are recently developed accessories, a detailed description of which must be sought for in the original publications.† The electrodes described by Ettisch and Péterfi are

* Florey, H. *Proc. Roy. Soc., B*, 1926, c, 269–273.

† Ettisch, G. and Péterfi, T. *Pflügers Arch. f. d. ges. Physiol.*, 1925, ccviii, 454.
Taylor, C. V. *Proc. Exp. Biol. & Med.*, 1925, xxiii, 147.
Taylor, C. V. and Whitaker, D. M. *Protoplasma*, 1927, iii, 1.

micropipettes of Jena glass filled with KCl-agar. A glass capillary is filled with the agar and one end sealed off in the flame. The other end is then drawn out into a micropipette and the agar in the capillary warmed so that it may expand and fill the pipette completely. At this moment the closed end is immediately broken. The agar will then remain in the tip of the pipette. Taylor describes a slightly different method for securing an agar filled pipette. He also gives a method for making platinum microelectrodes. A platinum wire (No. 35 C.P.) is inserted into a closely fitting quartz capillary and the two together drawn over a minute oxy-acetylene flame to an insulated needle point. The method of securing a good platinum tip at the end of the quartz glass capillary, as described in his original publication, has been revised recently. Since quartz and platinum do not have the same coefficient of expansion it is difficult to draw them out together in the flame. Therefore it has been found necessary, after drawing, to cut back the quartz from the tip by means of a micro-diamond cutter mounted on the micrurgical apparatus and operated in the field of the microscope. The projecting platinum filament is then melted into a pear-shaped bulb which is drawn tightly into the quartz capillary by the contraction of the platinum core. The flame for this purpose is operated in the field of the microscope.

Micromagnets, (p. 67) consisting of quartz with a soft iron core are drawn in the same manner as the platinum electrodes. The very tip of the iron core is completely enclosed by the quartz. The free end of the iron wire which projects beyond the base of the shank is soldered to the soft iron core of the magnet spool.

The micromagnets and the microelectrodes are supported and operated in the moist chamber by means of the micromanipulator.

III. Applications of the Micrurgical Technique

1. Protozoology and Embryology. This technique is particularly well adapted to experimental work on protozoa and on the ova and blastomeres of developing embryos.

Rapidly swimming forms can be made temporarily motionless by means of narcotics, e. g., chloretone or magnesium sulphate. Frequently shreds of cotton spread on a coverslip suffice to keep some organisms in a restricted field so that they may be seized with the microneedles.

For a wholesale cutting of some protozoa and ova the free hand method (p. 39) is entirely satisfactory. However, when the direction of the cut is to be specially oriented or when pieces are to be cut the micrurgical instrument is far more satisfactory. One needle should be more or less vertical and the other should have its shaft bent on itself so that the tip is almost horizontal (p. 56). The vertical needle is to hold the cell and to move it into the desired position. The horizontal needle is the cutting instrument. When

cutting a cell in two one must take into account the tendency of many cells to lose their more or less fluid contents if their surface membrane is torn. In the cutting process the diagonally directed shaft of the needle is raised into the hanging drop until its tip touches the undersurface of the coverslip with its shaft extending under the cell in the direction of the proposed cut. The needle is then raised still more. Its tip remains more or less in position while the elasticity of the glass allows the shaft immediately back of the tip to rise and cut into the cell as the latter is pressed against the undersurface of the coverslip. In this way the ever-deepening constriction in the cell eventually cuts it into two intact pieces.

Nuclei and other cellular organellae may be dissected out or cut with the microneedles. Solutions of various substances can be injected into the cytoplasm or the vacuoles and material can be sucked out of cells with the micropipettes.

Difficulty may be experienced with organisms and ova possessing tough pellicles. In developing ova the investing fertilization membrane can be removed, by sucking the still unsegmented eggs into a mouth pipette, the capillary aperture of which is larger than the diameter of the egg but smaller than that of the fertilization membrane. The same result may be obtained by straining the eggs through silk bolting cloth with meshes of the proper size.

2. Cytology and Histology. Histological material of both plants and animals offers abundant material for micrurgical studies. During the microdissection of intracellular structures care must be taken to distinguish between the physical state and appearance of the structure before and after death of the cell. In somatic tissue cells the observer soon learns to appreciate certain characteristics of the living cell, e.g., a peculiar translucency of the cytoplasm and especially of the nucleus. The nucleus is usually the first structure to exhibit visible death changes.

Chromosomes are readily obtained for dissection from the pollen mother-cells of certain plants* and germ cells of insects.† The 3 mm. water immersion apochromatic objective is very good for this work. The pollen mother-cells of Tradescantia are about 65 microns in diameter. An anther of a young flower bud is crushed between two coverslips in a drop of equal parts of 10 per cent saccharose and of plant sap. The coverslips are then separated and one is inverted over the moist chamber (cf. p. 49), on the stage of the microscope.

The testicular follicles of insects (grasshoppers, crickets, cockroaches) are placed in a drop of body fluid or blood serum. A few follicles are then cut off and transferred to a coverslip where they are carefully torn with needles. The germ cells float out free in the drop. The preliminary teasing

* Chambers, R. and Sands, H. C. *J. Gen. Physiol.*, 1923, v, 815.

† Chambers, R. General Cytology, Chicago, 1924, Sect. v.

should be done in a moist atmosphere (see moist chamber for teasing, p. 49). For a discussion regarding artificially made physiological fluids the reader is referred to a review article by Dittler.*

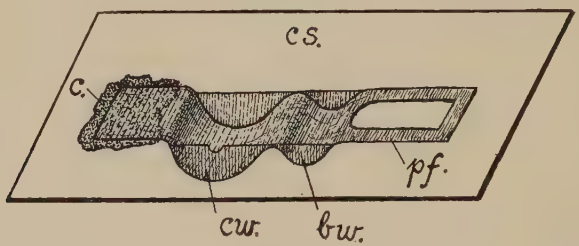


Fig. 19. Dr. Renyi's celluloid chamber cemented at one end, c., to the undersurface of a coverslip for holding a tadpole. c.s., coverslip; c.w., chloretone well; b.w., well for body of tadpole; p.f., perforated flap to hold tail against coverslip.

An ingenious contrivance for holding a living tadpole in place so that the cellular tissue of its tail can be experimented upon with microneedles has been devised by Dr. Rényi of the University of Pennsylvania (personal communication) (Fig. 19). Strips of thin celluloid are cemented together to form a well held to the coverslip at one end and with an extending horizontal flap at the other. By raising the flap the body of the tadpole can be slipped into the well which holds it snugly with the tail of the tadpole extending flat between the coverslip and the flap of the well. The flap is perforated so that when the coverslip is inverted and mounted on the moist chamber the under surface of the tail is exposed and can be microdissected. An additional well which is in communication with the well containing the body of the tadpole contains a drop of chloretone (1–5000) to narcotize the tadpole.

For streaming protoplasm the unicellular root hairs of seedlings and the stamen hairs of Tradescantia are to be recommended.

The stretched-out tongue of a pithed frog or the intestinal mesenteries of frogs and small mammals and the mesonephros of amphibia offer excellent opportunities for both microdissection and injection.

It is also possible to work with surviving tissues of amphibia and mammals, e. g., ciliated epithelium, gastric glands, muscle, etc., etc. Ova isolated in the fluid from the follicles of mammalian ovaries can be microdissected with ease.

3. Tissue Culture. The micrurgical method lends itself very well to operating on cells in tissue culture. Several articles have already appeared on results obtained from such operations and recently a detailed description of the special technique involved has been given by Péterfi.† Péterfi

* Dittler, R. *Handb. biol. Arbeit. meth.* (Abderhalden) 1922, *Abt.* v, i, 379.

† Péterfi, T. *Arch. exper. Zellforsch.*, 1927, iv, 165.

discusses the construction of a moist chamber in which manipulations are possible with a maintenance of a constant temperature and a prevention of infection. An adaptation of the Carrel culture tubes to this method is also described.

4. Cellular Physiology. The fresh water ameba has proved to be excellent material for studies on the effect of electrolytes on protoplasm, the colorimetric determination of the hydrogen ion concentration and the oxidation reduction potential of protoplasm.*

For experiments of this kind it is essential to have a cell in which moribund changes are easily recognizable. Favorable material includes ciliated cells in active movement, active muscle, especially of Amphibia, and marine ova.

The comparison of color tints of injected cells with standard colors in buffer solutions can be done by projecting the image of the standard colors into the field of the microscope. For this purpose is to be recommended the excellent microcolorimeter devised by Vlès.† This is a contrivance which casts a beam of light through a colored filter on a prism mounted in a special form of an ocular. It is manufactured by Nachet Cie, Paris. Pantin‡ describes a very good method in which colored test tubes are placed between the source of illumination and the mirror of the microscope. By moving the substage condenser the image of the various colored test-tubes can be focussed in the microscopic field. It is possible to use this method also by means of a camera lucida. The Needhams§ use a set of micro test-tubes, viz., glass capillary tubes, filled with dyes and placed on the coverslip in the field of the microscope. Another good method for comparing colors injected into a cell with a standard color filter is to use two microscopes furnished with a comparison ocular. This is an ocular mounted on the middle of a horizontal cylinder, the two ends of which project downward and are inserted in the drawtubes of the two microscopes. On the stage of the one microscope is the series of the standard color filters and on that of the other is the moist chamber with the cells to be microinjected.

The micrurgical method has also proved valuable for a study of the physical state of protoplasm. This and other methods, some of which are more strictly quantitative, have been reviewed in considerable detail by Weber.‖

5. The Darkfield. Microdissection and injection in the dark field has been made possible by the use of a special substage condenser of Péterfi¶ manufactured by Zeiss. There are two forms of condenser, one with a free

* See articles on micrurgical studies in *J. Gen. Physiol.* from 1926 and after.

† Vlès, F. *Compt. Rend. Soc. de Biol.*, 1926, xciv, 879.

‡ Pantin, C. F. A. *Nature*, 1923, cxi, 81.

§ Needham, J. and Needham, D. M. *Proc. Roy. Soc., B*, 1926, xcix, 383.

‖ Weber, F. *Handb. biol. Arbeitsmeth.* 1923, (Abderhalden) *Abt.* 11, ii, 655.

¶ Péterfi, T. *Ztsch. wiss. Mikroskop.*, 1926, xliii, 186.

working distance of 9.4 mm. (N.A. 0.4) to be used only with low power objectives and a second with a working distance of 4.5 mm. (N.A. 6.5) to be used with medium objectives. Leitz is manufacturing one for microdissection under high powers.

6. Isolation of Micro-organisms. Among the first to devise mechanical contrivances for isolating bacteria are Schouten* and Barber. It was the use of Barber's Pipette Holder which first opened up the possibilities of microdissecting and injecting living cells.

For those who are interested in the technique for isolating bacteria, reference is made to the articles of Schouten, Barber, Kahn,† Chambers‡ and Wright (p. 45). The first four references deal with the use of a single pipette holder. Wright advocates the use of a double manipulator. His procedure, in brief, is as follows:

One pipette is filled with the dilute suspension of the organisms from a young liquid culture. The second pipette is filled with sterile broth. With the first pipette minute droplets are blown on the undersurface of the roof of the moist chamber. As soon as a droplet is found which contains only a single organism the second pipette is immediately brought into the field and the droplet sucked into it. This pipette is now removed from the apparatus and the inoculation made on an agar slant or a fluid culture medium.

7. Microscopic Microchemistry.§ The micrurgical technique enables one to handle and mix micro-droplets (approximating 5 to 10 cubic micra) of chemical solutions in the field of the microscope. For this purpose two microinjection instruments should be mounted on the manipulator.

The coverslips for roofing the moist chamber (p. 49) may be of glass or of mica and should be perforated with a hole of about 18 mm. in diameter. The boring may be done by mounting the slip on a metal plate with balsam and cutting with a cork borer on a lathe. The edge of the borer should be covered with oil containing pumice powder.

Experiment 1. The mixing of two liquids, one of which is the unknown solution and the other a reagent:

A drop of the unknown is suspended from the coverslip roof of the chamber. A drop of the reagent is then placed on a square slip (the reagent slip) which is inverted over the hole in the roof and some of the reagent sucked into one micropipette (pipette R). This pipette is then lowered below the surface of the hanging drop and the chamber is moved by means of the mechanical stage until the drop of the unknown is brought into the field. Some of the unknown is sucked into the second pipette (pipette U) which is then lowered. The next step is to move the chamber again until a dry portion of the reagent coverslip is brought into view. Pipette R is now raised carefully until it touches the undersurface of the slip, a microdroplet of the reagent is ejected and the pipette lowered. Pipette U is now moved into place and raised into the microdrop, a small amount of the unknown discharged and the pipette immediately withdrawn. In this manner the operator has the

* Schouten, S. L. *Konigl. Akad. Wetensch., Amsterd. Proc. Sect. Sc.*, 1911, xiii, 840.

† Kahn, M. C. *J. Infect. Dis.*, 1922, xxxi, 344.

‡ Chambers, R. *J. Bacteriol.* 1922, viii, 1; *J. Infect. Dis.*, xxxi, 334.

§ This section was contributed by H. Pollack of my laboratory.

advantage of observing the actual mixing of the fluids and is able to follow the successive steps of the reaction.

The reagent slip can now be removed by means of a pair of forceps and another slip with a drop of the next reagent substituted. An entire qualitative analysis can thus be performed on one drop, about $\frac{1}{30}$ c.c., simply by drawing small portions of the drop into a micropipette and testing these very small fractions. The reaction may be performed under as high a magnification as desirable.

Experiment 2. Dark-field observations can also be made by means of Péterfi's substage condenser described on page 47.

Experiment 3. Decantation may be done by sucking the liquid from a precipitate with one pipette, washing with liquid from the second pipette and again sucking up the wash water with the first pipette. It is always advisable to have a few drops of distilled water suspended from the roof for washing the pipettes.

Experiment 4. For dialysis a collodion membrane can be made to cover the tip of a micropipette. For this purpose a pipette should be selected having a shaft about three inches long. Before mounting the pipette in the injection apparatus its tip is broken by rubbing against glass until the aperture is of the desired size. It is then dipped into the liquid against which the solution to be tested is to be dialyzed. By capillarity a sufficient amount of the liquid is drawn in. The open end of the shaft of the pipette is now sealed off in a microflame, after which the tip of the pipette is dipped into collodion and quickly withdrawn. Before the collodion dries on the tip the shaft must be rotated between the operator's fingers to expand the air inside the shaft and prevent the collodion from being sucked into the pipette. The process should be repeated two or three times in order to insure having a good membrane over the tip. When this is done the excess shaft including the sealed end is cut off and the prepared pipette mounted in the injection apparatus in the manner described on page 57.

Experiment 5. Colorimetric determination of the pH of a liquid. The coverslip should contain a series of drops of different indicators, a drop of the unknown and a few drops of distilled water. Droplets of the unknown and of the indicator may be mixed according to the procedure in Experiment 1.

CHEMICAL AGENTS: VITAL STAINS

NATHAN CHANDLER FOOT, M.D.

Introduction 74. Routes of injection 74. Types of material used 75. Specificity of vital dyes 75. Applicability of vital staining 76. Technique of vital staining 76. Fixation of vitally stained tissues 79. Combinations of vital stains 80.

I. Introduction

The term "vital staining" is somewhat misleading; "vital" it certainly is, being performed *intra vitam*, but it is hardly "staining" in the usual sense of that word. If the reader expects to obtain diffuse cytoplasmic and precise nuclear coloration by this method, as one would with the more usual technique, he will be disappointed. Vital staining applies to a specific coloration of the cytoplasm of certain cells, chiefly those of the "reticuloendothelial system" and, so far as we are concerned, consists of a process of ingestion of particles, either solid or colloidal, by these cells. There has been much speculation as to how the cytoplasm becomes vitally stained. Hypotheses have been advanced in favor of a specific affinity of preexisting granules in the cytoplasm for these dyes, of the filling of vacuoles by the particles of coloring matter, or of a simple process of phagocytosis of particulate or colloid material. It is unimportant to us which of these is the true solution of the problem, although the last two are probably nearer the truth than is the first.

In employing vital stains, the syringe and needle are the only apparatus needed. Naturally, aseptic technique is imperative. In using these stains intravenously an added precaution is necessary since one must guard against the presence of particles, or clumps of coloring matter large enough to form emboli in the capillary vessels. The use of normal salt solution as a suspending fluid is usually absolutely interdicted, particularly if benzidine dyes or other colloidal suspensions are to be employed, because it clumps the particles and brings about vascular occlusion with results that are usually as prompt as they are fatal.

II. Routes of Injection

Vital dyes may be injected into any part of the body, depending upon what is desired; they are usually used: 1. **Intravenously,** 2. **Intraperitoneally,** or 3. **Subcutaneously.** The dosage in the last two methods is of less importance than in the first; rather large doses are necessary to bring about complete results in their case, while smaller doses are needed with intravenous administration. These are the common routes, but vital dyes may be injected into other cavities or systems of the body if so desired, such, for instance, as the pleural sac or the trachea.

III. Types of Materials Used

The substances used for vital staining may be divided into four chief classes:

1. **Simple suspensions of particulate material,** such as lampblack, India ink, carmine, indigo carmine, graphite, metals, etc.

2. **Colloidal suspensions of similar substances,** Higgins' ink, lampblack and gelatin, lithium carmine, etc.

3. **Colloidal suspensions of silver or gold,** or their salts, particularly albuminates such as protargol, argyrol.

4. **Colloidal suspensions of various benzidine dyes,** such as trypan blue, Niagara blue, isamin blue, pyrrhol blue, trypan red, Janus green, etc.

The choice of one of these depends largely upon the desire of the experimenter and the nature of the experiment to be performed. Sometimes one method, sometimes another, will prove most satisfactory; they may often be combined, as in the instance of experiments to determine the origin of the "dust cells" of the lung, where one dye was injected into the trachea and the other through the blood stream.* Sometimes it is merely a question of convenience, some dyes being readily available, while some are not.

IV. Specificity of Vital Dyes

1. **Phagocytes.** If one desires to stain the phagocytes, almost any one of the above mentioned materials may be used, injected either directly into the immediate vicinity of the cells, or into the peritoneum, pleural cavity, or vascular system. The phagocytes comprise several types of cells that have been given a variety of names, according to their morpholgy—endothelial leucocytes, monocytes, clasmatocytes, histiocytes, etc., etc.

2. **Leucocytes.** Many of the dyes (particularly colloidal carbon) will, when injected intravenously, be taken up by the monocytes; the polymorphonuclear leucocytes rarely ingest any but the benzidine dyes and they do this usually only under circumstances indicating stagnation of the circulation. They are sometimes seen stained in thrombi or in pneumonic exudates, for example. In this instance, however, the dye stains the granules of the leucocytes quite brilliantly, which points to a difference in the staining mechanism and suggests that its entrance into these cells is not purely a matter of ingestion. Lymphocytes and erythrocytes do not stain with these dyes.

3. **Connective Tissue.** The fibroblasts are sometimes vitally stained, taking up most of the materials enumerated if given sufficient time. There are various ways of explaining this, but they would lead us far afield.

* Permar, H. H. *J. Med. Research,* 1920, lii, 9.
Foot, N. C. *J. Exper. Med.,* 1920, xxxii, 533.

4. Vascular Endothelium. As a rule, this is not stained by the dyes we have already discussed, with the exception of colloidal carbon in suspension, as in Higgins' ink. Sometimes, in cases of stagnation, it may become colored by other materials, but this is unusual. Inflamed vascular endothelium is regularly impregnated by colloidal carbon and this fact is often overlooked; swollen, inflamed endothelium probably becomes sticky and the carbon particles adhere to its surface and gradually become taken up by the cytoplasm. The Kupffer cells are regularly stained with all of the vital dyes, a fact that tends to support the contention that they differ somewhat from ordinary vascular endothelium.

5. Epithelial Cells. Vital stains sometimes enter epithelial cells to a rather marked extent. If the staining be pushed beyond certain limits almost any of these substances will find their way into the liver cords. The benzidine dyes and lithium carmine, which are regularly excreted through the kidneys, stain the epithelium of the convoluted tubules. Otherwise the vital dyes do not appear in epithelium with any regularity.

Other Tissues. The various tissues of the body not already enumerated, do not become stained with these pigments or dyes.

V. Applicability of Vital Staining

This method is of the greatest value in investigating the phagocytic cells of the body, wherever they may be situated; the reticuloendothelium is readily identified by means of lithium carmine or the benzidine dyes, as are the histiocytes, or wandering connective tissue cells. The vascular endothelium may be investigated and identified by means of colloidal suspensions of carbon. The excretory and absorptive areas of the kidney epithelium may be mapped out by employing lithium carmine.* As the bone marrow contains much reticuloendothelium and many phagocytic cells, it may be vitally impregnated with colloidal carbon, become quite black and opaque, and its distribution in cancellous tissue be studied in three dimensions by clearing the bone by the Spalteholtz method.† The benzidine dyes may be used in embryology to study the absorption of the amniotic, or other fluids, by introducing them into the proper sacs.‡

VI. The Technique of Vital Staining

1. Particulate Matter. Various particulate substances and pigments may be suspended in distilled water, filtered through coarse filter paper to remove the coarser granules, sterilized and used for injection into the vascular system, body cavities, or subcutaneous tissue. Heidenhain (Schmorl cit., 1921)§ recommended a 0.4 per cent suspension of indigo carmine

* Suzuki, T. Nierensekretion, Jena, 1912.

† Wislocki, G. B. *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 132.

‡ Wislocki, G. B. *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 93.

§ Schmorl, G. Die pathologisch-histologischen Untersuchungsmethoden, Leipzig, Ed. 10–11, 1921.

for intravenous injection, 35 c.c. to 60 c.c. being administered to rabbits, 150 c.c. to 1500 c.c. to dogs, according to their size. For intraperitoneal, intrapleural or subcutaneous injection the amount depends upon the size and species of the animal used and should be determined by experimentation. Karsner and Swanbeck* used suspensions of carmine or lampblack for intrapleural injection in cats in amounts varying from 15 c.c. to 25 c.c. For subcutaneous injection the capacity of the areolar tissue to take up the dye is the best indication of the amount to be used, and this varies with the location—very little can be injected into the external surface of a rabbit's ear, but a good deal can be injected under the loose skin of the back. Any insoluble pigment may be used. Slavjansky† employed double injections (vascular and intratracheal) of particulate indigo and cinnabar as early as 1869.

Drinker and Shaw‡ have introduced a method of using suspensions of fine particles of manganese dioxide in acacia water for investigating the phagocytic power of the endothelium of various organs in different animals; the manganese is injected and then each organ is assayed about one hour later, to determine its manganese content. The particles may be seen under the microscope, in the endothelial cells, but this is a chemical test, rather than a staining method and the reader is referred to their article for further details.

2. Colloidal Suspensions of Particulate Matter. Particulate matter may be rendered colloidal by suspension in solutions of acacia or gelatin, when it becomes more precise in its action and acquires an affinity for the monocytes and vascular endothelium. This is particularly true of colloidal carbon, because particulate carbon, unaided by any emulsifying agent, is quite unsafe for this purpose. This should always be borne in mind.

Higgins' Ink. Higgins' waterproof black drawing ink, which is (in all probability) a suspension of finely-divided carbon in acacia water, with a little camphor as a preservative, is the most readily available colloidal carbon for laboratory use. It is necessary merely to dilute it with an equal volume of sterile distilled water in a sterile test-tube and it is ready for use. It is well to warm it to body temperature before injecting it. The average intravenous dose for rabbits is 5 c.c., repeating this daily for three or four days and then every three days thereafter. The ear is shaved, rubbed with alcohol and a marginal vein used as the most accessible site for injection. If the vein be too small to be entered readily by the needle, a little xylol rubbed over the skin will cause the veins to become congested and easily entered; of course, a little practice is necessary. Injections should be begun near the tip of the ear and each subsequent injection should be given a short distance nearer the head, in the direction of the blood stream; this makes

* Karsner, H. T. and Swanbeck, C. E. *J. Med. Research*, 1920, xlii, 91.
† Slavjansky, K. *Virchow's Archiv. f. path. Anat.*, 1869, xlviii, 326.
‡ Drinker, C. K. and Shaw, L. A. *J. Exper. Med.*, 1921, xxxiii, 243.

it possible to use the same vein several times before it becomes thrombosed. A sharp needle, introduced as short a distance as possible into the lumen of the vein, is an added precaution against thrombosis; do not injure the endothelium if it can be avoided. The use of this ink was introduced by George Wislocki of the Johns Hopkins Medical School, where it has been in use by various investigators.

Colloidal Lampblack. If one desires to make up colloidal carbon suspensions in the laboratory, this may be done by McJunkin's method.* Grind up a good quality of commercial lampblack in a mortar for half an hour and add 0.4 gm. of this to 100 c.c. of distilled water that contains 2 per cent gelatin. Sterilize and use as in the case of Higgin's ink (p. 77). The gelatin may be used in 1 per cent suspension and a small quantity of this added to 5 gm. of lampblack, little by little, as the latter is ground to a fine paste (Simpson, 1922†). This is then made up to 100 c.c. with 1 per cent gelatin suspension. McJunkin advises administering 5 c.c. to 9 c.c. of 10 per cent sodium citrate solution at the same time the carbon is injected, claiming that he thus produces an added specificity and affinity of the monocytes for the carbon.

Both of these methods are specific for monocytes and for vascular endothelium, particularly in inflamed areas. Ordinary particulate suspensions will not be taken up in the same way. It has been noted by Lang that colloidal suspensions of carbon become agglutinated when mixed with blood, thus becoming "a common suspension of rather coarse carbon particles." Be this as it may, the effects of injected particulate carbon are quite different from those of colloidal carbon. For a full discussion of this subject, the reader is referred to papers by Lang‡ and Wislocki.§

Lithium Carmine. This is a colloidal suspension of carmine, first devised by Ribbert (Schmorl, p. 76) for studying kidney function, and largely employed and popularized by Suzuki (p. 76), Aschoff and Kiyono‖ and Kiyono.¶ A concentrated suspension of carmine rubrum optimum (5 gm.) in cold saturated lithium carbonate is filtered and sterilized and 5 c.c. to 10 c.c. injected intravenously, in the case of rabbits. It must be slowly administered. It is well tolerated by most animals, but has been found very toxic in one species, the Japanese waltzing mouse and its hybrids. This should be taken into account if that animal is the subject of experimentation.

3. Colloidal Suspensions of Silver and Gold. Protargol was used intravenously by Askanazy,** Miller,†† and others at one time. Simpson has

* McJunkin, F. A. *Arch. Int. Med.*, 1918, xxi, 59.
† Simpson, M. J. *J. Med. Research*, 1922, xliii, 77.
‡ Lang, F. J. *Arch. Pathol.*, 1926, i, 41.
§ Wislocki, G. B. *Am. J. Anat.*, 1924, xxxii, 423.
‖ Aschoff, L. and Kiyono, K. *Folia Haematol.*, 1913, vi, 213.
¶ Kiyono, K. Die vitale Karminspeicherung, Jena, 1914.
** Askanazy, M. Aschoff's Pathologische Anatomie, Jena, 1923, i, 183.
†† Miller, J. *Beitr. z. path. Anat. u. z. allg. Path.*, 1902, xxxi, 347.

employed "red gold" (40 per cent gold in 60 per cent sodium lysalbinate), for staining the monocytes and Kupffer cells. These substances present no advantage over the other methods and have not gained enough popularity to warrant a discussion or description in this section. References have been supplied.

4. Colloidal Suspension of Benzidine Dyes. Vital staining with these substances was first discovered by Goldmann,* who obtained them from Ehrlich's laboratory. They were introduced into the United States by H. M. Evans† who employed them in a number of investigations. Their chemistry is fully discussed in an article by Evans and Schulemann.‡

As already stated, there are a number of these dyes, most of which are more or less toxic, so that it is best to limit oneself to those of proved worth, namely trypan blue and its American prototype Niagara blue 2b. (N.A.C. Co. Buffalo, N. Y.), both of which are as nearly innocuous as foreign material introduced into the circulation could be.

For use, they are dissolved (suspended) in sterile distilled water, 1 gm. per 100 c.c. and used immediately, for it is unsafe to use electrolytes, such as normal saline or Ringer's solution, and it is dangerous to allow the suspensions to stand for more than an hour or so, for they are apt to become agglutinated and become highly toxic. They may be used intravenously, subcutaneously, or injected into body cavities; the dosage for subcutaneous administration in rabbits, is 1 c.c. per 20 gm. body weight; for intraperitoneal injection, 10 c.c. to 15 c.c.; for intravenous administration, 5 c.c. to 10 c.c., slowly injected. The suspension should be brought to body temperature before injection. The day following the injection the skin takes on a distinctly bluish tinge and, if the injections be repeated several times, the animal becomes quite blue, skin and mucous membranes having become deeply stained, and excretes bright greenish-blue urine. If a red dye be preferred, lithium carmine may be used, or trypan red; but the latter is distinctly more toxic than trypan blue and should be administered at half the concentration of that dye.

VII. Fixation of Vitally Stained Tissues

For material stained with particulate material and the colloidal suspensions of carbon or metals, any fixation is appropriate, but if lithium carmine or the benzidine dyes have been used, they will lose in intensity if Zenker's fluid be employed for fixation, on account of the acetic acid it contains. Neutral 10 per cent formalin (4 per cent formaldehyde) undoubtedly gives the best results. If a chromium fixative be desired, however, Helly's solution (Müller's fluid 90 per cent, strong neutral—40 per cent formaldehyde—formalin 10 per cent) gives excellent results, although there is some paling of the blue dyes which become slightly greenish. The material is most satisfactorily cut in paraffin, although frozen sections or celloidin sections give good results.

* Goldmann, E. E. *Beitr. z. klin. Chir.*, 1909, xliv, 192.
Ibid., 1912, lxxviii, 1.

† Evans, H. M. *Am. J. Physiol.*, 1915, xxxvii, 243.

‡ Evans, H. M. and Schulemann, W. *Science*, N. Y., 1914, n.s., xxxix 443.

VIII. Combinations of Vital Stains

As might be supposed, it is possible to combine two or more types of vital stains if so desired. For example, one may inject colloidal carbon intravenously and administer Niagara blue intraperitoneally at the same time, using lithium carmine intrapleurally if so indicated. Some cells will ingest one dye, some another and others (the endothelial phagocytes, for example) may take up all three. The histiocytes or reticuloendothelial cells tend to take up the benzidine dyes and lithium carmine quite early, the carbon appearing later; or, a dye of one color may be administered intravenously and one of another hue given intratracheally, for experiments on the pulmonary circulation, etc. The experimenter may thus devise a number of useful combinations.

CHEMICAL AGENTS: SUPRAVITAL STAINS

FLORENCE R. SABIN

Supravital dyes 81. Methods of staining 82. Application of supravital stains 83.

I. Supravital Dyes

The supravital technique consists in studying cells immediately after they have been taken from the body, by means of their reactions to certain dyes. Of these dyes there are two groups: the first, of which vital neutral red,* is the type, react to several kinds of substances within cells; the second, of which Janus green is representative, respond to one substance only.

1. Dyes Reacting to Several Substances. In the first group, there are a number of different dyes, all of them weak bases,† methylene blue GG, azure, brilliant cresyl blue, etc., but neutral red is by far the most useful. In the first place, it is the least toxic, and in the second place, it has the advantage of being a chemical indicator within certain limits. Its color toward the acid end of its range, pH 6.8, is a scarlet red, while at the alkaline end, pH 8, it is a clear yellow.

It was shown in 1886–88 by Campbell‡ that the nuclei of certain plant cells could be stained supravitally with mauvein, methyl violet, and dahlia without interfering with cellular division or with cytoplasmic streaming, but with the cells of mammals, any staining of the nuclei or of the basic cytoplasm indicates a damage to the cell and vitiates the method.

Neutral red reacts to four different types of substances: First, certain granulations which are the products of cytoplasm. These are the specific granulations of the white blood cells, neutrophilic, basophilic, eosinophilic, pseudoeosinophilic, and amphophilic, together with certain secretory granules, for example, those of the islet cells of the pancreas.§ Second, the fluid of the so-called vacuoles of digestion of phagocytic cells. The dye becomes adsorbed by debris within these vacuoles. Third, the canalicular apparatus of the parietal cells of the gastric mucosa.‖ Fourth, the basophilic substance of young red blood cells as precipitated into the so-called reticulation.

2. Dyes Reacting to One Substance. Of the dyes of the second group, vital Janus green for mitochrondria is the best known. Bensley (1911–12) has shown that pyronin, 1 to 1000, can be used to bring out the blind ducts

* Conn, H. J. Biological Stains. Geneva, 1925.
Phillips, M., and Cohen, B. *Stain Technol.*, 1927, ii, 17.

† Irwin, M. *J. Gen. Physiol.*, 1926, x, 75.

‡ Campbell, D. H. *Untersuchungen aus dem Tübinger Inst.*, 1886–88, ii, 569.

§ Bensley, R. R. *Am. J. Anat.*, 1911–12, xii, 297.

‖ Harvey, B. C. H., and Bensley, R. R. *Biol. Bull.*, 1912, xxiii, 225.

of the islets of the pancreas and that methylene blue injected into the blood vessels of the pancreas in strength of 1 to 10,000, brings out characteristically the centroacinar cells. The number of these specific reactions will undoubtedly be greatly increased with further experimentation.

II. Methods of Staining

1. Preparation of Dry Color Films.* Dry films of brilliant cresyl blue were used by Levaditi† in 1901, but in concentrations that killed the cells. The technique was fully developed both with neutral red and with neutral red-Janus green and applied to blood and connective tissues by Simpson‡ in 1921 and by Sabin§ in 1923. This technique of the dry films, has the advantage of allowing the cells to be studied in their own fluids and can be applied not only to cells of the blood, lymph, and exudates, but to all tissues as well unless they are too densely fibrous. The stain is used in the form of a dry, even film on a slip. The first requisite in this technique is to have chemically clean slips.

To prepare the stain, no directions of the exact amount of dye can be given since this will differ slightly with every sample of dye. The amounts used are so small that it is more convenient to make the dilute solution from a saturated solution rather than by weight. Neutral red is dissolved in absolute alcohol, which must be neutral and so must have been distilled from lime. The slightest acidity in the alcohol will vitiate the vital reaction; failure to use absolute alcohol distilled from lime has been responsible for many failures with the technique.

To make the solution, to 10 c.c. of neutral absolute alcohol add from 30 to 50 drops of a saturated alcoholic solution of the dye. To find the exact number of drops, the blood of a rabbit makes a convenient test, since it has abundant basophiles and eosinophiles. In correct dilution, the granules of the basophiles will be a scarlet red and those of the eosinophiles a clear yellow or at most a slight coppery tinge. It is better to make up only 5 or 10 c.c. of the dilute solution at a time for any grease will spoil the entire sample. The stains must be kept only in glass stoppered bottles, preferably stoppered with the glass pipette, but great care must be used not to let the dye run into the rubber nipple.

To prepare the slides, hold the microscopical slip obliquely over the bottle of stain and flood its surface evenly with the dye, using great care not to let the dye touch the fingers, and letting the excess run back into the bottle as quickly as possible; stand the slip upright on an absorbent surface until dry; mark the stained surface with a glass pencil. Simpson's technique of pushing two or three drops of stain over the slip by the edge of another, as for blood films, is excellent. The saturated solution of neutral red, the dilute solution, and the dry films on the glass all keep indefinitely, but the slips must be carefully guarded from dust.

* Neutral red iodide prepared by the methods of Phillips and Cohen is being made by the National Aniline and Chemical Company, New York City and by Hynson and Westcott, Baltimore, Maryland.

† Levaditi, C. *J. de physiol. & de path. gén.*, pt. 1, 1901, 424.

‡ Simpson, M. E. *U. Cal. Pub. in Anat.*, 1921, i, 1–11.

———. *Ibid.*, 1922, xliii, 77.

§ Sabin, F. R. *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 277.

To make the films of Janus green and neutral red, have a stock solution of saturated Janus green in neutral alcohol. This solution is stable. Take 2 c.c. of the dilute neutral red and to this add 3 drops of the saturated Janus green. This solution will precipitate on standing and hence must be put onto slips at once. The mixture is stable in the form of the dye films. The range of strength of Janus green is from 3 to 6 drops of the dye per 2 c.c. of the alcohol. For tissues in which there is a great excess of young cells, the double strength is better. Above this strength the cells will be damaged by this dye. Janus green may also be used alone in the dry film.

2. Injection. Immediately after killing the animal, the vital dyes may be introduced through the blood vessels, dissolved in physiological saline in appropriate dilution, 1 to 10,000 for neutral red and 1 to 15,000 for Janus green. But this procedure has the disadvantage of introducing an accessory fluid, and the cells suffer a period of asphyxia before observation, the effects of which are difficult to estimate. However, for certain purposes it is the method of choice.

The vessels should first be washed out with salt solution to limit as much as possible a precipitation of the dye. It is well known that in the study of the subcutaneous tissue it is necessary to produce an edema before the supravital preparations can be made. This can be induced either by prolonged injection of the entire animal through the aorta or locally with a hypodermic injection of the physiological solution of the dye. Bits of the edematous tissue may then be mounted on dry films of neutral red, of the combination of neutral red and Janus green, or of Janus green alone. With the method of injection Bensley was able to demonstrate all the islet tissue of the pancreas, or to bring out the relation of the blind ducts to the islets by a combination of pyronin followed by Janus green, or to show the entire extent of the centroacinar cells as they can be shown in no other way.

With the introduction by Sherwood* of a method for fixing films of cells which had been supravitally stained with neutral red; and by Cash† and by Gardner‡ of methods for sections of tissues after they had been vitally stained by injecting the same dye into the blood vessels, the reactions of cells to such staining will become better known. With the use of injection, the strength of the dye, at least 1 to 1500, which seems necessary to insure permanence of the preparations, is at the upper limit of the physiological range and hence the reaction is not as sensitive as with the dry film method. For a fuller discussion of injection methods see the section by Foot in this chapter, p. 74.

III. Application of Supravital Stains

1. Blood. To make a preparation of fresh blood on dry films of stain, the size of the drop is important and can be learned only by experience. The drop must spread evenly and must be large enough to occupy the space between slip and cover without overlapping of the red cells. As soon as the blood has spread, seal the edges of the coverslip with vaseline of a

* Sherwood, M. B. *Proc. Soc. Exper. Biol. and Med.*, 1925–1926, xxiii, 622.

† Cash, J. R. *Proc. Soc. Exper. Biol. and Med.*, 1926, xxiv, 193.

‡ Gardner, L. U. *Proc. Soc. Exper. Biol. and Med.*, 1927, xxiv, 646.

melting point sufficiently high so that it will not be softened in the warm box; the vaseline is applied conveniently from a syringe with a cut needle. Place the preparation in the warm box, which is kept at 37°C. by electric lights, controlled with a regulator; it is interesting to note that if quickly sealed the cells will live for at least fifteen to thirty minutes at room temperature so that there is time to take the preparations from hospital to laboratory. The preparations with neutral red alone can be counted for about two hours; with Janus green the cells die in about half an hour.

a. Red Blood Cells. In the supravital films with neutral red, it is possible to make a reticulated red count, since the dye acts exactly as does brilliant cresyl blue. This technique has, however, no advantage over the well known method with brilliant cresyl blue, which involves fixing the stained reticulation. A fixed preparation of reticulation can also be made with neutral red. In two regards, however, the supravital technique is valuable in connection with the red cells. Fragmentation of red cells is very obvious in fresh blood; but since, as was shown by Rous and Robertson,* it is increased by temperature, the point must be carefully controlled with the thermometer. Indeed, the amount of fragmentation of the red cells serves as a good check on the regulator of a warm box. The last stage of reticulation in the red cell is indicated by one or two small neutral red bodies. This reaction corresponds probably to the Isaacs granule and is the most sensitive indication of the beginning of regeneration of red cells in the marrow; with the supravital technique, this reaction is easily seen and cannot be confused with any other reaction.

b. White Blood Cells. The neutrophilic, pseudoeosinophilic, and amphophilic granules of the leucocyte stain characteristically in neutral red. The color reaction for all these types is a deep pink tone varying with the animal and with the age of the cell in each form. The reaction of these granules is more intense in the stage of the myelocyte, and becomes gradually less as the cell approaches senility. At the stage of senility, the granules of the neutrophiles lose their power to react to neutral red and become highly refractile, and the cell passes into the so-called non-motile phase. It has been shown by de Aberle† that all the discrepancies concerning the proportion of leucocytes to lymphocytes in rodents' blood can be cleared up by using the supravital technique, because the specific granulation of the leucocytes is so clearly visible. Besides the specific granulation, leucocytes frequently have vacuoles which stain in neutral red and indicate phagocytosis. These vacuoles always show a different shade of neutral red, a little more toward the acid range of the dye, and they are usually a little larger than the granules. They may become quite large. Besides the granules and vacuoles that react to neutral red, the leucocytes have mitochondria, usually rather small in size, which can be seen unstained or can be brought

* Rous, P., and Robertson, O. H. *J. Exper. Med.*, 1917*a*, xxv, 651; 1917*b*, xxv, 665.

† de Aberle, S. B. *Am. J. Anat.*, 1927, xl, 219.

out with Janus green. In the older leucocytes these grow fewer and fewer until they disappear in the senile forms. All these granules and vacuoles flow ceaselessly in the streaming of the cytoplasm which accompanies locomotion. In the supravital preparations the leucocytes are in constant ameboid locomotion, up to the non-motile phase of the senile type. The supravital technique does not bring out the nuclei as sharply as the fixed film and hence an Arneth count can be made more quickly with fixed films; it, however, does allow many qualitative observations on the leucocytes which are not possible in fixed films.

c. Basophiles. The granules of the basophiles stain the scarlet or acid reaction of neutral red. The granules are not quite uniform in size and a few of them react more intensely to the dye than others; this is especially true with the basophile of human blood. In all forms the cell shows active ameboid movement. The presence of vacuoles cannot be discriminated.

d. Eosinophiles. The large refractile granules of the eosinophiles react characteristically in neutral red, with the alkaline reaction of the dye. In most samples of neutral red the reaction is a coppery red but the perfect reaction is a clear yellow. Very rarely, the shade of reaction will vary slightly between two eosinophiles in the same drop of blood. Occasionally, there are a few tiny basophilic granules to be seen in eosinophiles, and this is the rule in the eosinophiles of the guinea pig. The eosinophiles are not commonly vacuolated. The technique is more sharply differential for eosinophiles than fixed films; as is well known, an occasional leucocyte in the fixed film has slightly larger granules than the average neutrophile and makes a form difficult to discriminate. The question does not arise with the supravital technique, for the dye as chemical indicator is differential.

e. Lymphocytes. The characteristic features of lymphocytes are the clear cytoplasm, the large content of mitochondria, usually in the form of rods, and the type of motility. Lymphocytes also show a few vacuoles, varying in number from one or two up to ten or twelve or more. Of the three forms, the small, intermediate, and large, the intermediate types are more often found moving, but McCutcheon* has shown that all lymphocytes will move if the preparation be allowed to stand long enough. The type of motility is characteristic; the nucleus is in the front end of the advancing cell and changes so markedly in shape as to seem to take part in the locomotion of the cell.

f. Monocytes. The monocytes have a denser cytoplasm than the lymphocytes and contain bodies, possibly vacuoles, that react with a constant salmon shade, probably near the neutral reaction of neutral red. The monocyte of the blood is a young form of cell, showing less complete maturation than the leucocyte, hence its strongly basophilic cytoplasm and the obviousness of its centrosphere. Usually the neutral red bodies are arranged around the centrosphere making a rosette, with abundant mitochondria

* McCutcheon, M. *Am. J. Physiol.*, 1924, lxix, 279.

in the periphery of the rosette. During motility, or through some damage to the cell, this arrangement may be interfered with. In the supravital films the cell moves slowly; the nucleus is in the center; and the cell is often stretched out in the form of a triangle. The preparation does not give sufficient space between slip and cover for the play of its surface film as demonstrated in the moving pictures of Carrel, Ebeling and Goldberg.

g. Leucemic Blood. In blood in which there are large numbers of immature cells, the mixture of neutral red and Janus green should be used. Janus green, however, limits motility to such an extent that such blood should also be studied in neutral red alone. The fact that the myelocyte does not show ameboid movement and that its granules stain more deeply in neutral red makes the discrimination of the myelocytes from the leucocyte easy with this technique. The proportion of mitochondria in immature cells and the exactness with which a few neutrophilic granules can be discriminated in the early myelocytes make this technique indispensable in the discrimination of the myeloblastic from the lymphatic forms of leucemia. In leucemic blood, there are conditions in which the cells are so fragile and so many of them make smudges in the technique of dragging the blood across slip or cover for the fixed film, that recourse must be made to the supravital technique, where the damage to fragile cells is much less.

As a routine in the study of blood, if differential counts are made with the supravital technique, it is well to take a pair of coverslips or a slip for the fixed technique to be stained with methylene blue azur and kept as a permanent record.

2. Cells in Fluids Other Than Blood Plasma. In the cerebrospinal fluid, the free cells tend to deteriorate more rapidly than in blood plasma, and it has been shown by Kubie and Shults* that the supravital technique allows a much sharper differentiation than other methods.

Material from exudates can be obtained through pipettes and mounted as blood films. Cells can be obtained from the peripheral sinuses of lymph glands by introducing a fine pipette tangentially to the gland; the supravital technique shows that this fluid is more toxic to the cells than the fluid of the central sinuses or of the thoracic duct.

3. Cells from Organs. The supravital technique is especially well adapted to the study of phagocytosis, inasmuch as the vital neutral red brings out the vacuoles of digestion and gives some indication of the degree of digestion of phagocytized material and the chemical reaction of the fluid. Thus, in the application of the method to organs, more can be learned concerning the cells of the connective tissues in organs than for the most part of parenchyma itself. The technique can be applied to tissues removed for diagnosis and to the organs from an autopsy. With experimental animals the material is, of course, studied at once; with human

* Kubie, L. S., and Shults, G. M. *J. Exper. Med.*, 1925, xlii, 565.

material the autopsy must be performed while the cells are still living, but it is interesting to note that the cells of the bone marrow survive longer than others and can be studied supravitally for six or seven hours after death.

With experimental animals some material can be obtained to advantage through capillary pipettes. This is true of material from the spleen, lymph glands, and bone marrow, if taken by punctures while the blood is still circulating. Material from organs is obtained either by gentle scraping of a freshly cut surface or from tiny pieces. In scrapings from the lungs, an increase in free phagocytic cells over the normal is readily demonstrated. A discrimination between clasmatocytes and the epithelioid cells of pathological reactions can be made out. Bronchial epithelium comes off in sheets and is told by the cilia; the epithelium of the air sacs is more rarely seen but is identified by its relation to a framework of elastic tissue. Elastic tissue does not come free to any extent from gentle scraping of the normal lung.

With the liver, the condition of the Kupffer cells is readily told, but too little is known of variations in the appearances of the liver cells with function to discriminate any but extreme abnormal changes. The same is true for the kidney. The supravital technique is especially well adapted to the study of spleen, lymph glands, and bone marrow. With the bone marrow, a tiny piece of tissue, the size of about two pinheads, should be cut with fine curved scissors, with no handling of forceps, and carefully transferred to a slip. With very gentle pressure on a coverslip the tissue is slowly flattened out; the main piece allows the tissue between the fat cells to be analyzed, while the free cells along the edges are like a blood film and allow of differential counts of the cells. A record of the level at which the marrow is functioning, both as regards the red cells and the white cells, and the proportion of red cell formation to white cells, made from the surveys with the fresh tissue, is of the utmost importance. With the same method, tissues from any organ can be studied unless the tissue is too fibrous. Kubie* has adapted the technique to a study of the tissues of the brain.

* Kubie, L. S. *J. Exper. Med.*, 1927, xlvi, 615.

CHAPTER III

BACTERIOLOGICAL METHODS

H. J. Conn, F. B. Mallory and Frederic Parker, Jr.

General Considerations 88. Study of Pure Cultures 88. Unstained preparations 88. Stained preparations 90. Study of Bacteria in Natural Habitat 100. In soil 100. In dairy products 101. Sectioned Material 102. Gram-negative bacteria 102. Gram-positive bacteria 105. Acid-fast bacteria 106.

A. GENERAL CONSIDERATIONS

The preparation of specimens for microscopic examination is simpler in the case of bacteria than in that of higher plants or animals, except when some special technique such as flagella staining is in question. It is, however, very important that a correct optical system be used; and the microscope should be given special consideration.

The microscopes most commonly used in bacteriological laboratories are equipped with Abbé condenser, triple nosepiece bearing 1.9 mm., 8 mm. (or 4 mm.) and 16 mm. objectives, with oculars of 6.4× and 10× magnifications. Little attention need be given to the selection of the lower-powered objectives; but the 1.9 mm. oil-immersion objective and the oculars should be carefully selected. The oil-immersion lens for best results should be apochromatic; but on account of expense and the care necessary in handling such an objective, the apochromatic type is ordinarily less practical than the fluorite objective of 1.9 mm. The latter, although having a lower numerical aperture than the apochromatic lenses, gives sufficient definition so that it may be used with quite high-powered oculars, and is entirely satisfactory for ordinary bacteriological work. If a fluorite objective is employed, the oculars for routine use may be 7.5× and 12.5×, instead of the 6.4× and 10×, because of the greater definition secured; and in this way greater magnification may be obtained. The oculars of 10× and below may be of the Huygenian type; but for best results the higher powered oculars should be of the compensating type. An ocular as high as 15× compensating may be used with a 1.9 mm. fluorite objective; and in the study of minute bacteria such a combination is often very useful. For routine work, however, the 6.4× and 7.5× oculars are most satisfactory.

B. STUDY OF PURE CULTURES

I. Unstained Preparations

1. Hanging Drop. Unstained preparations of bacteria are generally utilized for determining whether a culture is motile. The important points to be observed in making them, therefore, are that the culture be at its

greatest vigor, and that the preparation be so made as not to hinder the motility of the organisms. Hanging-drop preparations are usually employed.

Cultures for this purpose should be inoculated into whatever medium, either liquid or solid, is understood to be most favorable for their growth. They should be incubated at optimum temperature until good growth has taken place, ordinarily about twenty-four hours. In the case of liquid cultures, a loopful should be placed on the coverslip; in the case of solid cultures (agar), the same method may be used if sufficient liquid has been exuded at the base of the tube, or if not, a minute portion of the surface growth may be mixed with a small drop of water on the coverslip. The coverslip bearing a drop of culture should be inverted over the hollow of a depression slip, with care to see that the drop does not touch the glass at any point. If a depression slip is not available, good results may be obtained by a slightly different technique which does not give true hanging-drop conditions. A cover glass is supported by two or three small pieces of a broken coverslip placed on an ordinary microscopical slip. If this latter technique is used, one does not need to take precautions to prevent the drop touching the glass; for motility can take place between slip and cover almost as freely as in a hanging-drop.

The preparation should be examined with a dry objective of about 4 mm. focus. If the bacteria are very small, a comparatively high-powered ocular (as for example 12.5×) may be used to give the desired magnification; but for ordinary purposes, an ocular of 6.4× or 7.5× is more satisfactory. Artificial light must be used, either with or without a "daylight" screen, and the diaphragm below the condenser should be nearly closed.

2. Study of Microscopic Colonies. There are three ways of making a microscopical study of unstained bacterial colonies: (1) direct examination on the culture plate; (2) transfer of the colony to a microscopical slip; (3) agar block or gelatin drop culture.

(1) Direct examination on a culture plate requires a rather low power and does not give very satisfactory results. It is ordinarily employed only when precautions must be taken to avoid contamination of the culture plate.

(2) Removal of the bacterial colony is accomplished by cutting out a block of the medium and lifting it up with scalpel or spatula. For this purpose the colony should preferably be growing in agar; removal of a gelatin colony for microscopical examination is a much more difficult procedure. The block of medium containing the colony should be placed on a slip with the colony to be studied at or near the top of the block. It may then be covered with a coverslip and examined with a relatively high power. An oil-immersion objective may sometimes be used to good advantage; but ordinarily the same combination of lenses should be used as mentioned in the case of hanging drop.

(3) The agar block or gelatin drop culture is a little more difficult to study. Slips with deep depressions must be used for this work and should be sterilized before using, together with coverslips to fit them. They may be sterilized inside of Petri dishes and kept inside of them until used. For an agar block culture, a small piece of sterile agar should be cut out of a Pétri plate in which it has been poured without inoculation. It should be cut and removed with a previously flamed scalpel or spatula, and transferred under aseptic conditions to one of the sterile coverslips. Just before placing on the coverslip, the culture to be studied should be inoculated on the surface at one minute spot, and the inoculated side should be placed against the coverslip. The coverslip bearing the block should then be inverted into the depression of the slip and should be sealed in such a way as to prevent evaporation. For sealing the coverslip in place, pyroxylin cement gives very good results.

The gelatin drop culture is managed a little differently. Ordinarily the inoculation is effected by placing a minute amount of bacterial growth on the sterile coverslip. A drop of sterile gelatin which has been melted and then cooled down almost to its solidifying point is placed on the spot thus inoculated. The drop of inoculated gelatin is covered with the depression slip and immediately transferred to a refrigerator or other cooling device until the gelatin solidifies; it is then sealed around the edges of the coverslip.

These agar block or gelatin drop cultures, after incubation (not higher than 20°C. in the case of gelatin), are to be examined by the same methods as just described for other unstained preparations.

II. Stained Preparations

1. Preparations for Staining. For a few purposes bacteria are stained without drying by adding dilute stain to the liquid in which they are suspended. They are then examined without washing, or else the washing is done beneath the coverslip by introducing water from one side and blotting the stain out from the other. Ordinarily, however, bacteria are stained in dried smear preparations.

Smear preparations are simply made by mixing a small amount of the bacterial growth (best taken from the surface of agar) with a drop of water (preferably distilled) on a slip. Care should be taken not to use enough of the bacterial growth to give noticeable turbidity. The drop is then spread over an area of one or two square centimeters, according to the amount of liquid in the drop, and is allowed to dry. No fixative is needed, because the bacterial growth ordinarily is sufficiently gelatinous in its nature to stick to the glass without the addition of any fixing agent. Mild heat is sometimes used so that bacteria will adhere more firmly, but it is not ordinarily necessary. The smears should never be heated above the boiling temperature.

Occasionally difficulty in obtaining a good smear preparation is experienced because of the presence of slimy substances produced by the bacteria. Smear preparations from such organisms cannot well be stained in the usual way, either because the entire mucous mass takes this stain and obscures the bacteria or else because nothing in the preparation stains. Such organisms are sometimes handled by centrifuging to remove the bacteria from the slime; the bacteria at the bottom of the centrifuge tube are then washed with water and smeared as usual on the slip. In the writer's experience, it has proved much simpler to make preparations from such organisms in the usual manner and then to stain with some special dye such as rose bengal (p. 100).

2. Staining Methods. The most recent summary for staining procedures for pure culture study of bacteria is part of the "Manual of Methods for Pure Culture Study of Bacteria," published by the Society of American Bacteriologists (Geneva, N. Y.) and issued by the Committee on Bacterial Technic. This summary of staining methods is given in Supplement A to the Manual entitled "Staining Suggestions," of which the second edition has been issued (March 1927). Permission has been secured from the committee to quote these staining procedures here. Attention, however, is called to the fact that the Manual of Methods is a loose-leaf publication constantly subject to revision, and that the committee assumes no responsibility for the methods being up-to-date for more than a brief period after the date of issue of each section of the publication. To obtain the most recently approved methods, therefore, the reader is referred to the publication in question which the committee plans to keep up-to-date.*

This supplement to the Manual of Methods, omitting the introductory paragraph, runs as follows:

In the literature concerning staining methods there is a surprising amount of inaccuracy. Few of the formulae found can be accepted in the light of present knowledge without some degree of interpretation to make them more explicit; in some cases the author's intentions in the matter are evident, in others they can be only inferred. Hence, in the following pages, two formulae for some of the staining solutions are given: the original one (or that generally found in text books); and the emended formula as interpreted by the committee. The latter is intended to give the same solution as probably employed in the first place; but the committee assumes no responsibility for the identity of the two and offers the emendation merely as an attempt to make the original formula more definite and more workable when using the stains and reagents at present on the market.

In using any of the methods it must be remembered that a blind adherence to a staining technique is no guarantee that the result will be satis-

* Committee on Bacteriological Technic. Manual of Methods for Pure Culture Study of Bacteria. Publ. by Soc. of Amer. Bacteriologists, Geneva, N. Y., 1923, with revisions of various sections at later dates. A loose-leaf publication.

factory. Even experienced workers sometimes discover to their dismay that they took too much for granted as to the purity of their reagents, cleanliness of slides* and covers, or proper compounding of the staining solutions. A technique should, therefore, be checked upon known organisms as controls. It is, furthermore, important to know that the solutions and water used for dilution are reasonably free from bacteria and their spores.

A. General Bacterial Stains.

(1) *Ziehl's Carbol-fuchsin*

Old Statement of Formula		Emended Statement of Formula†	
		Solution A	
Sat. Alc. Sol. basic fuchsin	10 c.c.	Basic fuchsin (90 per cent dye content)‡	0.3 gm.
5 per cent sol. carbolic acid	100 c.c.	Ethyl alcohol (95 per cent)	10 c.c.
		Solution B	
		Phenol	5 gm.
		Distilled water	95 c.c.
		Mix Solutions A and B.	

(2) *Loeffler's Alkaline Methylene Blue*

Original Statement of Formula		Emended Statement	
		Solution A	
Conc. Sol. methylene blue in alcohol	30 c.c.	Methylene blue (90 per cent dye content)	0.3 gm.
Sol. KOH in distilled water (1:10,000)	100 c.c.	Ethyl alcohol (95 per cent)	30 c.c.
		Solution B	
		Dilute KOH (0.01 per cent by weight)	100 c.c.
		Mix Solutions A and B	

This formula is still very useful, although no longer as necessary as formerly. The use of a weakly alkaline solution was apparently to overcome the acidity of poorly neutralized dyes formerly on the market and to secure partial polychroming of the methylene blue. The methylene blue at present available, however, especially that which is certified by the Commission on Standardization of Biological Stains, does not require neutralization, and contains a sufficient admixture of the lower homologues of methylene blue (azure, etc.) to give good differentiation without treatment with alkali. As a result, good results can ordinarily be obtained if distilled water is substituted for the dilute alkali in the above formula.

*"Slide" in this quotation is equivalent, in most cases, to the term "slip," used elsewhere in this book.

† The emended statements of the formulae in the following pages are not formulae especially recommended by the committee; but represent an attempt to put the original formulae in more up-to-date form and to show what the original author probably intended. The committee assumes no responsibility for the actual correctness of its interpretation. Corrections and comments from users are invited.

‡ It is not necessary that dry stains of the exact dye content specified be used in this or in the following formulae. Samples of higher or lower dye content may be employed by making the proper adjustment in the quantity used.

(3) *Aniline Gentian Violet (Ehrlich)**

Original Statement of Formula		Emended Statement	
		Solution A	
Sat. Alc. Sol. Gentian violet	5–20 c.c.	Crystal violet (85 per cent dye content)	2.5 gm.
Aniline water (2 c.c. aniline shaken with 98 c.c. water and filtered)	100 c.c.	Ethyl alcohol (95 per cent)	12 c.c.
		Solution B	
		Aniline	2 c.c.
		Distilled water	98 c.c.
		Shake and allow to stand for a few minutes, then filter.	
		Mix Solutions A and B.	

The use of crystal violet in this formula is recommended because this dye is more uniform than the various products sold as gentian violet. The committee, however, does not recommend the use of an aniline formula in the Gram stain, but rather one of the two given below.†

B. The Gram Stain. There are a large variety of modifications of the Gram stain, many of which have recently been listed by Hucker and Conn. Two of the more satisfactory of the recent modifications may be mentioned.

(1) *Hucker Modification (recommended by the Committe)*

Ammonium Oxalate Crystal Violet

Solution A

Crystal violet (85 per cent dye content)	4 gm.
Ethyl alcohol (95 per cent)	20 c.c.

Solution B

Ammonium oxalate	0.8 gm.
Water	80 c.c.

Mix Solutions A and B

Lugol's Iodine Solution

Iodine	1 gm.
Potassium iodide	2 gm.
Water	300 c.c.

Counterstain

Safranin (sat. solution in 95 per cent alcohol)	10 c.c.
Water	100 c.c.

Technique. Stain one minute with the gentian violet solution; wash in water; immerse in iodine solution for one minute; wash in water and blot dry; decolorize in 95 per cent alcohol for thirty seconds with gentle agita-

* Although various aniline water formulae for this dye are known as Ehrlich's, he seems properly to be credited only with the idea of using aniline water in the formula. Various subsequent authors have modified the solution to suit themselves; and as a result the amount of gentian violet recommended in different places varies to the extent shown in the left-hand column above.

† Loeffler, F. *Mitt. a. d. k. Gesundheitsamte,* 1884, ii, 421.
Ziehl, F. *Deut. Med. Wchnschr.,* 1882, viii, 451.

tion; cover with counterstain for ten seconds; then wash dry, and examine with oil-immersion lens.

(2) *Kopeloff and Beerman Modification (recommended by the Committee)*

Alkaline Gentian Violet

Solution A

Gentian or crystal violet*	1 gm.
Water	100 c.c.

Solution B

Sodium bicarbonate	1 gm.
Water	20 c.c.

Just before use, mix 30 drops of solution A with 8 drops of solution B in a beaker.

Iodine Solution

Iodine	2 gm.
Normal solution sodium hydroxide	10 c.c.

After the iodine is dissolved make up to 100 c.c. with water.

Counterstain

Basic fuchsin	0.1 gm.
Water	100 c.c.

Technique. Stain five minutes or more with the alkaline gentian violet solution; rinse with the iodine solution; add fresh iodine solution and allow to stand two minutes or longer; drain off iodine solution and blot dry (without washing); decolorize with 100 per cent acetone, adding drop by drop to the slip while tilted until no color is seen in drippings (generally less than 10 seconds); dry in the air; counterstain for ten to thirty seconds; wash in water, dry, and examine with oil-immersion lens.†

C. Acid-fast Staining. Various methods have been given for determining the acid-fast properties of an organism; but all are really variations of the same general procedure: staining deeply with carbol fuchsin, then decolorizing with acidified alcohol, followed or accompanied by a counterstain.

(1) *The Ziehl-Neelsen method*‡ is: carbol fuchsin with gentle steaming for three to five minutes or cold for fifteen minutes; wash in water; decolorize in 95 per cent ethyl alcohol containing 3 per cent by volume concentrated hydrochloric acid until only a suggestion of pink remains; wash in water; counterstain with saturated aqueous methylene blue or Loeffler's methylene blue; wash and dry.§

* The authors specify either crystal violet or methyl violet 6B. Probably any of the gentian violets now sold under the Commission certification would be satisfactory; they are ordinarily either crystal violet or one of the bluer grades of methyl violet.

† Hucker, G. J. *Abstr. Bact.*, 1922, vi, 2.
Hucker, G. J. and Conn, H. J. *N. Y. Agr. Exp. Sta., Tech. Bull.* 93, 1923.
Kopeloff, N. and Beerman, P. *J. Inf. Dis.*, 1922, xxxi, 480.

‡ See Stitt's Practical Bacteriology, Blood Work, and Animal Parasitology. Ed. 7, Phila., 1923, p. 58.

§ Compare with method given in Mallory's section on Acid-fast Staining of Sections (p. 94).

(2) *Mallory and Wright** recommend the following: carbol fuchsin with gentle steaming for one to four minutes; wash in water; decolorize in 70 per cent ethyl alcohol containing 1 per cent by volume of concentrated hydrochloric acid until red disappears (not more than a few seconds); wash in water; wash in 95 per cent alcohol for thirty seconds; wash in water; counterstain for thirty seconds with Loeffler's methylene blue solution; wash and dry.

(3) *The Spengler method*† calls for: carbol fuchsin with gentle steaming for three to five minutes; without washing apply for two to three seconds a mixture containing equal parts of 96 per cent ethyl alcohol with 5 per cent picric acid (the latter having been allowed to stand twenty-four hours and then filtered before adding to the alcohol); apply 3 to 4 drops of 15 per cent nitric acid; after five seconds pour off nitric acid, and apply picric-acid-alcohol again until yellowish; wash and dry.

D. Spore Stains. It must be understood that the procedures for acid-fast staining ordinarily work as spore-stains. Most of the methods for staining spores differ only in the manner of applying heat during the staining and in that the acid used in decolorizing is diluted with water instead of with alcohol. The method of Dorner‡, however, depends upon a somewhat different principle and is very much more simple in its manipulation.

(1) *Dorner's Method (recommended by the Committee)*

(*a*) *For bacteria not forming slime:*

(1) Make a heavy suspension of the organism in 2 to 3 drops of distilled water in a small test-tube.
(2) Add an equal quantity of freshly filtered Ziehl's carbol fuchsin.
(3) Allow the mixture to stand in a boiling water bath for ten to twelve minutes.
(4) On a coverslip or slide mix one loopful of the stained preparation with one loopful of a saturated aqueous solution of nigrosin.
(5) Smear as thinly as possible and do not dry too slowly.

(*b*) *For bacteria forming slime:*

(1) Make the suspension in 0.5 c.c. broth.
(2) Add 1 c.c. of ordinary nutrient gelatin (melted).
(3) Add 1 c.c. of carbol fuchsin and stain as above.
(4) Wash out the colloids with warm water, with the help of centrifuge and sedimentation.
(5) Mix with nigrosine and proceed as above.

E. Flagella Stains. Flagella staining is an extremely difficult technique and there have been more different methods proposed for the purpose than for any other bacteriological procedure. There is no evidence that any one of these procedures is universally better than any other. The important thing is that one method be selected and that the user become so familiar with it that he can obtain good results; although it is probable that some

* Mallory, F. B. & Wright, J. H. Pathological Technic, Ed. 8, Phila., 1924, p. 393.
† See Stitt, p. 94.
‡ Dorner, W. *Jahrb. d. Schweiz.*, 1922, xxxvi, 595.
Dorner, W. *Le Lait*, 1926, vi, 8.

kinds of bacteria stain better by one method, others by some other. Some of the favorite formulae are as follows:

(1) *Loeffler's Flagella Stain**

(1) Mordant:

Solution of tannic acid (20 per cent in water)	10 c.c.
Sat.† aqu. solution of ferrous sulphate	5 c.c.
Sat. solution of basic fuchsin in 95 per cent ethyl alcohol (i. e. about 3 to 5 per cent)	1 c.c.

(2) Stain:

Carbol fuchsin.

There are various modifications of this technique. Duckwall,‡ for example, has modified it by using 15 c.c. of a 13.3 per cent solution of tannic acid. The mordant is ordinarily used fresh, and filtered each time before using; although Kulp§ obtains better results by letting it stand over night and centrifuging before use. Kulp prefers gentian violet to basic fuchsin both in the mordant and in the stain.

Shunk's‖ modification differs so radically from the original that it must be treated separately. It is as follows:

(2) *Shunk's Flagella Stain*

(1) Mordant:

Solution A

Sat. aqu. solution of tannic acid	30 c.c.
Solution of ferric chloride (5 per cent in water)	10 c.c.

Solution B

Aniline oil	1 c.c.
Ethyl alcohol (95 per cent)	4 c.c.

Solution A is best prepared a week or more ahead of time and filtered before use. When using, place about 8 drops of solution A on the slide and immediately add one drop of solution B; and there is then a precipitation on the slide. The excess mordant is carefully drained off, and the stain applied without previous washing.

(2) Stain:

Carbol-fuchsin, 1 per cent safranine in 50 per cent alcohol, aniline gentian violet, or Loeffler's methylene blue may be used. Shunk recommends the following, however:

Leoffler's methylene blue solution	30 c.c.
Solution B of Shunk's mordant	3 c.c.

This solution is immediately ready for use and keeps well.

*Loeffler, F. *Centbl. f. Bakt.*, 1890, vii, 625.

† By "Sat. solution" (unless otherwise specified) is meant a solution saturated at room temperature (21° to 25°C.).

‡ Duckwall, E. W. Canning and Preserving of Food Products with Bacteriological Technic. Pittsburgh, 1905, 458 pp.

§ Kulp, W. L. *Stain Technology*, 1926, i, 60.

‖ Shunk, I. V. *J. Bact.*, 1920, v, 181.

(3) *Casares-Gil's Flagella Stain**

As Published by Plimmer and Paine (p. 98)

(1) Mordant:

Tannic acid	10 gm.
Aluminum chloride (hydrated)	18 gm.
Zinc Chloride	10 gm.
Rosaniline hydrochloride†	1.5 gm.
Alcohol (60 per cent)	40 c.c.

The solids are dissolved in the alcohol by trituration in a mortar, adding 10 c.c. of the alcohol first, and then the rest slowly. This alcoholic solution may be kept several years. For use, dilute with 4 parts of water, filter off precipitate and collect filtrate on the side allowing it to act for sixty seconds.

N. B. It has recently been stated (Thatcher‡) that better results can be obtained in this technique if the mordant is diluted only 2 to 1 instead of 1 to 4. Subsequent investigation by the committee (with the corroboration of Miss Thatcher) shows a dilution of 1 to 2 to be rather more satisfactory.

(2) Stain: Carbol fuchsin.

(4) *Gray's Flagella Stain*§

(1) Mordant:

Solution A

Potassium Alum. (sat. aqu. solution)	5 c.c.
Tannic acid (20 per cent aqu. solution)	2 c.c.
(A few drops of chloroform must be added to this if a large quantity is made up.)	
Mercuric chloride (sat. aqu. solution)	2 c.c.

Solution B

Basic fuchsin (sat. alc. solution)	0.4 c.c.

Mix Solutions A and B less than twenty-four hours before using. Both solutions separately may be kept indefinitely, but deteriorate rapidly after mixing.

(2) Stain: Carbol fuchsin.

The general method of applying flagella stains requires first the procuring of very young and vigorous cultures. The exact method of preparing these cultures varies with different organisms; but, in general, growth from twelve to twenty-four hours old on surface of agar gives best results. If old stock cultures are used, it is often necessary to make daily transfers for a few days before studying, in order to restore their vigor. Kulp (p. 96) obtains especially good results by using agar slants with plenty of condensation water; then from a twenty-four hour culture a loopful of the condensation water is transferred to a second agar slant, from which, twenty-four hours later, 1 or 2 drops of condensation water are poured off aseptically into a tube of sterile distilled water which has been kept at the same tem-

* See Galli-Valerio, B. *Centbl. f. Bakt.*, I *Abt. Orig.*, 1915, lxxv, 233.

† The authors specify rosaniline hydrochloride. There are, however, other basic fuchsins more universally available which ought to prove equally satisfactory.

‡ Thatcher, L. M. *Stain Technology*, 1926, i, 143.

§ Gray, P. H. H. *J. Bact.*, 1926, xii, 273.

perature as the culture; the tube is then incubated at the optimum temperature of the organism for from forty-eight to seventy-two hours. A similar procedure was recommended some time ago by Johnston and Mack.* Others, however, seem to find that bacteria shed their flagella if left so long in distilled water.

A very important point is that the cover glasses or slides used be scrupulously clean. They should be prepared by treating in a hot cleaning fluid consisting of sulfuric acid and sodium or potassium bichromate made up according to the formula as given in standard texts. (N.B. After this fluid becomes green or brown it should be discarded.) For best results the covers or slides should subsequently be cleaned in strong caustic soda or potash, rinsed in weak hydrochloric acid, and then in distilled water. They should finally be placed in alcohol and kept there until ready for use. With cover glasses the alcohol can be burned off just before use, while holding each cover with a forceps. In the case of slides, Plimmer and Paine† recommend baking on a wire gauze over a Bunsen burner just before using.

The growth should be prepared by diluting considerably in sterile distilled water, and then carefully placing tiny drops or streaks on the coverslip, discarding those coverslips which show signs of grease. The drops or streaks should be so thin as to dry almost instantly; and no appreciable heat should be used in drying them. Some writers recommend drying at body heat, others at room temperature. Fixation of the films by flaming is sometimes specified, but is not to be recommended as it destroys the flagella if they are allowed to become too hot.

Plimmer and Paine recommend the use of slides (scrupulously cleaned), instead of covers. The slide is heated before use and cooled to about body temperature. Then a drop of the culture fluid from a 3 mm. loop is placed at one end of the slide, the slide tilted to allow the suspension to run down the slide. The slide should be warm enough to allow rapid drying. Gray also uses slides, placing a large loopful of the culture suspension near one end and making the film by spreading the fluid gently over the slides with a strip of unsized paper (e. g. typing paper).

After the dried films on the cover glasses or slides are ready, the mordant is applied (filtered before use if this is called for by the technique adopted). The mordant is allowed to act between half a minute and three minutes, the time differing with different organisms. In the case of Loeffler's mordant, gentle heat is called for, sufficient to allow visible steam to rise from the solution. The mordants of Casares-Gil, Shunk and Gray are applied cold; Gray allows the mordant to act for ten minutes, although the committee

* Johnston, O. P. and Mack, W. B. *American Medicine*, 1903, vii, 754. (Original not seen.) Reference obtained from Moore and Hagan's Laboratory Manual in General and Pathogenic Bacteriology and Immunity (1925), p. 99.

† Plimmer, H. C. and Paine, S. G. *J. Path. Bact.*, 1921, xxiv, 286.

has obtained very satisfactory results with half that period. After rinsing off the mordant the stain is applied in the same way, using gentle heat (enough to cause steaming) in the case of Loeffler's method, but no heat in the case of the other methods given above. The stain is applied from one to three minutes or more. In general, the procedures calling for heat specify about one minute, the others three minutes. Plimmer and Paine recommend five minutes, Gray five to ten minutes.

Other methods of manipulation are sometimes called for, such as dropping the coverslips into test-tubes containing the steaming solutions or by using slides and allowing them to rest on a water bath in which the water is a little below boiling temperature. In this matter, also, the best procedure is undoubtedly the one with which the user is more familiar.

Poor results may be caused by greasy cover glasses or slides, cultures too old or growing on an unsatisfactory medium, allowing the bacteria to stand too long in the dilution water before drying, too thick suspensions of the bacteria, insufficient or excessive mordanting, under-staining, or over-staining. It is very difficult for the beginner to tell which of these points is at fault in a given case, and unless he has expert assistance he can do nothing but proceed by the method of trial and error until chance brings him the right combination. It can be said in general, however, that poor results are more likely to be caused by improper handling of the culture, or by greasy coverslips or slides than by the use of an unsatisfactory staining procedure.

The influence of the personal equation is so great in all this work that the committee hesitates to recommend any one procedure. In the chairman's laboratory, best results have been obtained with the Casares-Gil and the Gray methods, the former showing more heavily stained flagella and the latter giving preparations with a clearer background. A second member of the committee, Wright, agrees with this but has also obtained splendid results with Shunk's technique, which has not proved successful in the chairman's laboratory. A third member of the committee, Kulp, as stated before, prefers a modification of the Loeffler procedure. There is little question but that other laboratories comparing the methods given above would come to still different conclusions.

F. Capsule Stains. Various methods of staining capsules are given in the literature. None of them, apparently, can be regarded as absolutely reliable. The two following seem to be most used at present in this country.

(1) *Hiss' Method**

Preparations from ascitic fluid or serum media, or other preparations mixed with serum before use, are dried on the slide or cover. The stain used consists of 5 or 10 c.c. sat. alc. gentian violet or basic fuschin made up to 100 c.c. with water. This is placed on the dried and fixed preparation and

* Hiss, P. H., Jr. *J. Exper. Med.*, 1905, vi, 317.

gently heated for a few seconds, until steam arises. The dye is washed off with a 20 per cent aqueous solution of copper sulfate (crystals). The preparation is then dried by blotting.

(2) *Huntoon's Method**

A 3 per cent solution (100 c.c.) of sodium caseinate ("nutrose") is cooked for one hour in flowing steam; 5 c.c. of 2 per cent phenol is added, and the fluid decanted into test-tubes. The organisms to be stained are mixed with a drop of this solution, spread in a thin film on a glass slide, and dried in the air without fixing.

The stain is:

2 per cent aqu. solution of phenol	100 c.c.
Concentrated lactic acid	0.25–0.5 c.c.
1 per cent acetic acid	1 c.c.
Sat. alc. solution of basic fuchsin	1 c.c.
Carbol fuchsin (old solution)	1 c.c.

The stain is kept on the film for thirty seconds, and the latter washed in water and dried.

g. Conn's Stain for Slime Forming Bacteria.† As explained before, the bacteria which form slime can be handled as ordinary dried smear preparations if the proper stain be used. For this purpose, one of the high fluorescein derivatives such as erythrosin, phloxine or rose bengal proves most satisfactory. The following formula is recommended:

Rose bengal (85 per cent dye content)	1 gm.
Phenol (5 per cent aqueous solution, by volume)	100 c.c.

This solution is applied cold or preferably with a little heat for about one minute. The slide is then washed very briefly and examined as usual.

C. STUDY OF BACTERIA IN THEIR NATURAL HABITAT

I. In Soil

Bacteria in soil may be stained by the following technique: One gram of soil is mixed with a sufficient quantity of a gelatin fixative to bring the total bulk up to 10 c.c. This fixative is prepared by dissolving 0.015 per cent of gelatin in boiling water and using after it is cooled. It is conveniently kept in plugged test-tubes containing 10 c.c. each, which can be sterilized and then used one by one so that the stock of the fixative solution does not become contaminated. The exact proportion of soil and fixative used is not important unless the technique is to be used in estimating the numbers of bacteria present.

About 0.01 c.c. of this infusion is placed on a slide. This quantity should be measured exactly by means of a capillary pipette if an estimate of num-

* Huntoon, F. M. *J. Bacteriol.* 1917, ii, 241.

† Conn, H. J. *Stain Technology*, 1926, i, 125.

bers is to be made; otherwise it may be withdrawn without measuring from the tube, by means of a wire loop about 2 mm. in diameter. The slip used should have been washed in alcohol so as to be moderately free from grease. By means of a stiff wire or needle, a drop of the infusion is smeared over the surface to cover an area of about a square centimeter. If a count of the bacteria is to be made this area should be accurately measured, by means of a square of the specified size placed underneath the slip. If no count is to be made the exact size is immaterial.

The preparation is immersed from one to three minutes in acetic acid, is then washed, and dried on a boiling water bath. While still thus heated, it is stained for about one minute with rose bengal. This staining solution may be of the formula given before (p. 100) or it may be a plain 1 per cent aqueous solution. Either phloxine or erythrosin may be used in place of rose bengal but the writer obtains better results with the latter.

The preparation should be examined with a 1.9 mm. fluorite or apochromatic objective and a 12.5× compensating ocular. If it is desired to count the bacteria, the ocular should contain a micrometer bearing a circle divided into quadrants by means of cross lines, and the apparent area of the circle should have been previously determined by means of a stage micrometer.

II. In Dairy Products

Methods have been proposed for observing bacteria both in milk and in cheese. The former has become a standard procedure and is therefore described at length below. The microscopical observation of bacteria in cheese, however, has been used only for experimental purposes, and is not detailed here. It is a method calling for imbedding in paraffin, sectioning, and staining by procedures that are essentially histological. It is described by Hucker.*

For staining bacteria in milk, 0.01 c.c. is withdrawn with a capillary pipette and smeared over one square centimeter of a glass slip as above described in the case of soil infusion. The size of the sample and the area of the smear need not be measured exactly unless the bacteria are to be counted.

The milk smears are dried either at room temperature or with gentle heat; the temperature of a boiling water bath is too high. The slides are immersed in xylol for one to two minutes to wash out the fat. They are then placed in alcohol for about the same length of time to fix the smear to the slip. They are then stained for about a minute in Loeffler's methylene blue solution (which may be prepared if desired with water instead of dilute alkali as a diluent). After staining they are decolorized in alcohol until the smear appears faint blue. After this it is ordinarily found that best results can be obtained by dipping momentarily into the methylene blue again and then washing with water. They are subsequently dried and examined

* Hucker, G. J. *N. Y. Agric. Exp. Sta., Tech. Bull.* 87, 1921.

under immersion oil. A lower-powered ocular can be used than in the examination of soil.* The ocular should contain a circle of known apparent area as described under soil examination. A fairly accurate estimate of the number of bacteria present in the milk can be made by counting from 10 to 100 fields (according to the abundance of the organisms) averaging the results and multiplying by $100 \times \frac{1}{\text{size of field, in sq. cm.}}$.

D. SECTIONED MATERIAL†

The stains most commonly used for demonstrating bacteria in sections are methylene blue, crystal, methyl or gentian violet and basic fuchsin. Various solutions of these dyes have been devised and are usually known by the names of the men who originated them. The fixatives recommended are alcohol, formaldehyde, corrosive sublimate and Zenker's fluid or combinations of certain ones of them. Zenker's fluid can be highly recommended partly on account of its excellent preservation of the organisms, but also because it furnishes the best fixation of the tissues and of the inflammatory exudation caused by the infectious agents.

From the point of view of staining, the gram-positive and acid-fast bacteria are the easiest to demonstrate because they can be colored differently from the surrounding tissue elements, in other words they can be stained differentially and hence stand out with great sharpness. The gram-negative organisms on the other hand are often difficult to demonstrate satisfactorily. As a result various staining methods have been devised both for this group of organisms and for different members of it.

The following methods can be recommended as the most generally useful and as probably the best which have yet been devised.

I. Gram-Negative Bacteria

1. Mallory's Eosin, or Phloxin, and Methylene Blue Stain. Zenker fixation, paraffin sections.

1. Stain sections in a 5 per cent aqueous solution of eosin or in a 2.5 per cent aqueous solution of phloxine for twenty minutes or longer. Sometimes it is advisable to obtain a more intense stain by placing the dish containing the sections in the paraffin oven for thirty to sixty minutes.
2. Wash off in water.
3. Stain in the following solution, diluted 1 to 10 for use, for thirty to sixty minutes:

Methylene blue	1 gm.
Borax	1 gm.
Water	100 c.c.

Pour the solution on and off the sections several times.

4. Wash in water.
5. Differentiate and dehydrate in a dish of 95 per cent alcohol, keeping the section in constant motion, so that the decolorization shall be uniform. Control the result under

* Breed, R. S. and Brew, J. D. *N. Y. Agr. Exp. Sta., Tech. Bull.* 49, 1916.

† Mallory, F. B. and Parker, Frederick, Jr.

the microscope. When the pink color has returned to the section and the nuclei are still a deep blue, finish the dehydration quickly with absolute alcohol.

6. Clear in xylol.

7. Mount in xylol balsam.

For celloidin sections use 95 per cent alcohol, blot, and pour on xylol; repeat the last two steps until the specimen is clear.

It is important to get a deep stain with eosin, because the methylene blue washes it out to a considerable extent. The eosin must be used first, because methylene blue is readily soluble in an aqueous solution of eosin, and therefore is quickly extracted if the eosin is used after it, while on the other hand eosin is very slightly soluble in an aqueous solution of methylene blue which is precipitated by any excess of eosin.

The success of this staining method has been found by Wolbach to depend on the presence of colophonium in the alcohol used for differentiation. This is present in alcohol obtained from the barrel, but not in alcohol preserved in glass. It must, therefore, be added. This is most easily done by keeping on hand a 10 per cent solution of colophonium in absolute alcohol, and adding a few drops of it to the alcohol in which the sections are differentiated. Wolbach has also shown that sections fixed in formaldehyde may be stained by this method, provided the amount of colophonium in the alcohol be increased to from 3 to 10 per cent.

It is sometimes advisable to stain the sections more deeply than usual with the methylene blue solution and then to decolorize for several minutes in a 1 to 1000 aqueous solution of acetic acid before dehydrating in alcohol.

2. Giemsa's Method for Staining Bacteria in Sections.

1. Fix pieces of tissue not more than 2 mm. thick in sublimate alcohol, consisting of two parts of a concentrated aqueous solution of corrosive sublimate and one part of absolute alcohol. The fixation requires at least forty-eight hours. The fixing fluid is to be renewed after twenty-four hours.

The tissue may remain for as long as three months in the fixing fluid without disadvantage if evaporation is prevented.

2. Dehydrate in graded alcohols and clear in xylol. Imbed in paraffin. The sections should not be over 4 μ thick—2 μ are better. The tissues must not be handled with metal instruments until after they have been cleared in oil of cedarwood.

3. Treat sections with xylol, followed by graded alcohols and water.

4. Ten minutes in a solution consisting of KI, 2 gms.; distilled water, 100 c.c.; Lugol's solution, 3 c.c.

Instead of this mixture, it is possible to use Lugol's solution only (1 to 3 c.c. of it mixed with 100 c.c. of water or 70 per cent alcohol), or tincture of iodine diluted with alcohol. The use of the weak alcoholic iodine solution is indicated when a more intense blue staining of the cytoplasm is desired. Treatment with the weaker iodine solutions demands naturally a longer time, twenty to thirty minutes.

5. Treat with 95 per cent alcohol until the yellow color is removed. After a quick wash with distilled water place sections for ten minutes in a 0.5 per cent aqueous solution of sodium hyposulphite, then five minutes in tap-water, and for a short time in distilled water.

6. Stain with freshly diluted Giemsa solution two to twelve hours or longer. The solution recommended for this purpose should be made up according to the following modified formula:

Azur II—eosin	3.0 gm.
Azur II	0.8 gm.
Glycerin	125.00 c.c.
Methyl-alcohol	375.00 c.c.

The dilution should be 1 drop to 1 c.c. of water; or for a longer period of staining, 1 drop to 2 c.c. of water. After the first half hour the staining mixture is to be poured off and replaced by fresh.

7. Wash in distilled water and dehydrate as follows

(*a*) Acetone 95 c.c. plus xylol 5 c.c.
(*b*) Acetone 70 c.c. plus xylol 30 c.c.
(*c*) Acetone 30 c.c. plus xylol 70 c.c.
(*d*) Xylol pure.
(*e*) Cedarwood oil.

8. Mount in cedarwood oil.

The duration of the treatment with *a*, *b*, and *c* depends upon the degree of differentiation required.

The distilled water used for diluting the staining fluid must be absolutely free from acid. The slightest trace of organic or mineral acids, or even the presence of a considerable amount of carbonic acid, spoils the staining. The distilled water may be tested and corrected for use as follows:

Place 30 c.c. of it in each of four flasks. Add 1 per cent solution of carbonate of sodium (Na_2CO_3), 1 drop to the first flask, 2 drops to the second flask, and so on. Then take 10 c.c. from each flask in a clean test-tube and add 2 or 3 drops of a fresh solution of hematoxylin in absolute alcohol, which should be pale yellow to nearly colorless. Stand against a white background, and the flask with the right reaction should take on a violet tinge after one to five minutes.

For bringing out certain granules, etc., in special objects a larger amount of alkali in the water is necessary. In this case add to 20 c.c. of the water, shortly before mixing with the staining fluid, an additional drop of alkali solution.

3. Wolbach's Modification of Giemsa's Stain.

Zenker fixation; paraffin sections, not over 5μ thick.

1. Xylol, alcohol, Lugol's solution; alcohol as usual.

2. Five-tenths per cent sodium hyposulphite to remove the last traces of iodine, ten to fifteen minutes.

3. Wash in running water ten minutes, followed by distilled water.

4. Stain in the following solution for twelve to eighteen hours:

Distilled water	100 c.c.
0.5 per cent sodium bicarbonate	2 to 4 drops
Reagent methyl alcohol	3 c.c.
Giemsa's solution	2.5 c.c.

The stain should be poured over the slides immediately after mixing, and should be changed twice during the first hour. The slides are then allowed to remain in the third solution for twelve to eighteen hours.

5. Differentiate in 95 per cent alcohol.

The procedure of differentiation really consists in removing the excess of stain to a point where good histological detail is secured. If the sections are too blue, a better balance may be secured by adding very small quantities of colophonium to the alcohol.

6. Dehydrate rapidly in absolute alcohol.
7. Clear in xylol and mount in oil of cedarwood.

4. MacCallum's Modification of Goodpasture's Method.

Fix in Zenker-formaldehyde solution. Use very thin paraffin sections.

1. Stain for ten to thirty minutes or more in Goodpasture's fuchsin solution.

30 per cent alcohol	100 c.c.
Basic fuchsin	0.59 gm.
Aniline	1 gm.
Phenol crystals	1 gm.

2. Wash in water.
3. Differentiate in formalin (40 per cent solution of formaldehyde). Only a few seconds are required. The bright red color changes to rose.
4. Wash in water.
5. Counterstain in a saturated aqueous solution of picric acid for three to five minutes until the section becomes purplish yellow.
6. Wash in water.
7. Differentiate in 95 per cent alcohol. The red reappears and some of it is washed out, as is also some of the yellow of the picric acid.
8. Wash in water.
9. Stain in Sterling's gentian violet for five minutes or more.
10. Wash in water.
11. Gram's iodine solution for one minute.
12. Blot dry without washing.
13. Clear in a mixture of equal parts of aniline and xylol until no more color comes away.
14. Clear in two changes of xylol.
15. Mount in xylol balsam.

Gram-negative bacteria red, gram-positive bacteria blue; tissue red and blue; fibrin deep blue.

II. Gram-Positive Bacteria

1. The Gram-Weigert Staining Method.

Preferably Zenker's fixation, paraffin sections.

1. Stain sections lightly in alum-hematoxylin.
2. Wash in running water.
3. 1 per cent aqueous solution of eosin, one to five minutes.
4. Wash in water.
5. Aniline methyl-violet, one-half to one hour.
6. Wash off with water.
7. Lugol's solution, one to two minutes.
8. Wash off with water.
9. Blot with filter-paper and dehydrate and clear in several changes of aniline and xylol, equal parts.
10. Wash off with xylol.
11. Mount in xylol balsam.

When this method is used for staining actinomycetes a saturated solution of eosin should be taken in order to stain the clubs of the organisms sufficiently intensely.

If the tissues have been fixed in alcohol or formaldehyde a carmine stain for the nuclei can be used in place of the alum-hematoxylin and eosin.

The MacCallum-Goodpasture method can also be used for this group of organisms.

III. Acid-Fast Bacteria

The tubercle and leprosy bacilli and a few other gram-positive organisms have the property, when once deeply stained by certain aniline dyes, of resisting decolorization by a mineral acid followed by alcohol. A variety of methods have been devised for staining this group of organisms but only the simplest and most generally reliable is given here.

1. **Ziehl-Neelson Carbol-fuchsin Method.** This is the staining solution most commonly used. It keeps exceedingly well.

Saturated alcoholic solution of basic fuchsin	10 c.c.
5 per cent solution of carbolic acid in distilled water	90 c.c.

2. **Ehrlich's Aniline Fuchsin Method.** It is claimed that this solution stains the bacilli more intensely than carbol-fuchsin, but it has to be used fresh because it deteriorates rapidly.

Saturated alcoholic solution of fuchsin	16 c.c.
Aniline water (made by shaking together 5 parts of aniline with 95 parts of water and filtering the resulting milky fluid)	84 c.c.

The method of using these two staining solutions is the same.

Zenker fixation preferable; paraffin sections.

1. Stain sections deeply in alum-hematoxylin.
2. Wash in water.
3. Stain in carbol-fuchsin cold over night or steaming for three to five minutes.
4. Wash off in water.
5. Dip in acid alcohol (hydrochloric acid 1 c.c. to 99 c.c. of 70 per cent alcohol) for thirty to sixty seconds.
6. Wash thoroughly in several changes of water.
7. Decolorize and dehydrate in 95 per cent alcohol followed by absolute.
8. Clear in xylol.
9. Mount in xylol balsam.

If celloidin sections are used, they should be attached to the slip by the ether vapor method after the staining with alum-hematoxylin. They can be cleared from 95 per cent alcohol by the blotting paper xylol method.

The leprosy bacillus is more difficult to stain than the tubercle bacillus because it does not retain the dye so well. If good results are not obtained by the preceding method the following procedure can be recommended:

3. Flexner's Method for Staining Leprosy Bacilli.

1. Stain in alum-hematoxylin so as to get a sharp nuclear stain.
2. Wash in water.
3. Carbol-fuchsin two to five minutes steaming, or thirty to sixty minutes cold.
4. Water.
5. Treat on the slip with iodine solution one-half to one minute.
6. Water.
7. Blot, clear and differentiate in aniline oil.
8. Xylol, xylol balsam.

The Gram-Weigert stain gives brilliant results provided the organisms are deeply colored to begin with but of course the method has no diagnostic value by itself.

CHAPTER IV

GENERAL BOTANICAL MICROTECHNIQUE

William Randolph Taylor

(Section on Fats by Sophia H. Eckerson)

Histological Methods 109. Collection, preservation and preparation 109. Reduction of material to physical proportions within range of microscopical observation 116. Preparation and staining of sections 129. Microchemical reactions in plant membranes 136. Mounting methods 137. Cytological Methods 144. Choice of methods and standards 144. Examination of living cells 151. Preparation of cytological material for fixation 153. Choice of the fixing fluid 155. Imbedding and staining 159. Cytoplasmic inclusions 159. Fats 160. Special Methods for Particular Plant Groups 164. Algae 164. Fungi 173. Bryophytes 176. Pteridophytes 176. Gymnosperms 177. Angiosperms 178.

This chapter is intended to be strictly supplementary to the first part of the whole book, where McClung follows specimens of animal tissue through the fundamental preparatory methods.

Both plant and animal materials, however, may be used for introductory training with equal facility. The beginning student should study the first part very thoroughly before consulting this botanical chapter, and all students should beware of assuming that stages in treatment are unessential if omitted or only casually treated here, because processes are not always given in full, but often only their distinctive features emphasized. Cross references to other paragraphs will enable the user to fill important gaps, but a thorough acquaintance with the first part is essential. Stains, for instance, are largely treated separately, and mitochondria and related structures in plants are found in a general chapter including both animal and plant material.

The present chapter treats, first, methods applicable to structures both histological and cytological, found generally in most plants, and then gives special directions which are of particular importance mainly in relation to special plant groups. It does not consider field collecting or the laboratory culture of organisms, and therefore cannot take the place of a Collector's Handbook, or an Encyclopedia of Microscopy. A casual worker will not always be able to refer from the index to a full discussion of the methods of preparing any given sample, for the intention is to offer information and explanations that will facilitate a training in technique rather than a compendium of procedures by which an untrained operator might be led (fallaciously) to expect that he could, by imitation, duplicate the results of a skilled research student. It is obvious that a broad treatment of this kind can be only a compilation colored by the author's opinions of relative values and needs, however extended his training. Originality of method is not to be sought here, but in research papers. The methods given are comparatively few, and a selection has been made of those that offer

advantages in simplicity or precision, discarding those of primarily historical interest or of marked undependability. Limitations of space would have forced this even if it had not been a virtue. The text is not designed for the reference of a skilled investigator, but to supply him his fundamental training, and, should he pass from a familiar to a new field of work, to give him methods by which he might be able to start his investigation and test his material. The most important collateral texts that a student should make available to himself are: Schneider-Zimmerman, Die botanische Mikrotechnik, 2^te^ Aufl., R. Krause, Enzyklopädie der mikroskopischen Technik, 3^te^ Aufl., 1926, continued, and C. J. Chamberlain, Methods in Plant Histology, Edition 4, in addition to works on the optics and operation of the microscope and its equipment.

A. HISTOLOGICAL METHODS

I. Collection, Preservation and Preparation

1. **The general sequence in plant histology** through which specimens pass involves their collection, the selection of the appropriate portions, then their removal, perhaps their preservation, followed by detailed manipulations such as sectioning, maceration etc., which prepare the material for study, and finally the mounting of the ultimate product on slips for observation. The methods suited for vascular plants will be considered here and other groups discussed in special paragraphs. There is little that calls for comment respecting the collection of the specimens in the field. It is obvious that great caution must be exercised to secure representative material in perfect condition. In all serious researches, herbarium specimens from the sources that gave the histological (or equally the cytological) material should always be preserved and filed in permanent collections to permit verification of the original determinations. In fact, portions of the identical specimens should be kept for these records whenever possible, and their relation to the portions studied should be correctly entered on the labels. All specimens, as collected, should be kept appropriately moist and aerated so that they may remain in good health until they can be studied or killed and preserved. Just before killing, the portions to be kept for study should be cut free of waste and should be immediately immersed in the preservative. For rough studies in anatomy most material can be sufficiently killed and preserved in 4 per cent formaldehyde solution. For more critical work the following may be used: 70 per cent alcohol without or with 10 per cent of glycerin, formol-alcohol, acetic-formol-alcohol, the mercury fluids, strong chrom-acetic and strong Flemming (p. 423 for formulas and after-treatment). This list gives the less critical fluids first. As one passes to the more precise in action the size of the pieces fixed should be decreased until in strong Flemming the diameter should not exceed 2 to 4 mm., but such fluids are generally used only in studies of tissue differ-

entiation, cell inclusions, etc. In general, this series passes from mixtures giving but poor preservation of the cell contents to ones which, while maintaining the interrelation of the cells, also show correctly the general internal cell structure. After being killed the material should be brought gradually into 70 per cent alcohol, except when one of the first three fluids, which are preservatives in themselves, are used. If the material is to be imbedded before cutting it should be prepared according to the schedules which are given with the imbedding methods (pp. 125, 126). Special preservation methods in histology are too numerous and for the most part too limited in applicaton to deserve consideration here. A few of wide application may be important.

Calcium carbonate or sulphate deposits on or in plant tissues do not usually lend themselves to the preparation of permanent mounts. Material should be fixed in alcohol free from acid. Sections of tissues containing, for instance, cystoliths as in *Ficus* or *Urtica* can be stained and dehydrated quickly, cleared and mounted in balsam, but it is difficult to avoid some solution of the cystolith, which will often dissolve if the sections are left for a little in tap water. (For cryptogams with considerable Ca_2CO_3 deposits see p. 112 and the paragraph on the group concerned p. 171.)

Cellulose, as food reserve-cellulose in seeds, often offers great interference to cutting if it is dry and mature. When the conditions of study permit, the material should be gathered and preserved before maturity has completely hardened the walls, and the sections should be cut under water rather than alcohol. Seeds of *Iris* or *Phoenix dactylifera* can usually be managed in this way, the walls appearing practically as thick as if dry seeds had been used. Otherwise, methods applicable to hard woods or coals must be adopted (pp. 113, 122).

Collenchymatous wall thickening and pectic jellies interfere with satisfactory cutting if they are too soft. Such material should be preserved and cut under 70 per cent alcohol, swelling if necessary with water afterwards. It would seem advantageous to harden with even stronger alcohol, but the collenchymatous material sometimes does not expand well after such treatment. In general, soft tissue, if it is to be cut unimbedded will behave best in alcohol (70 to 85 per cent), especially if it has stiff bundles scattered through soft areas, as is often the case with monocotyledonous plants and ferns.

Crystals as cell inclusions (usually calcium oxalate, occasionally the sulphate) show by far the best in fresh-cut sections from living material. However, they may be mounted in 2 per cent acetic acid and will keep for a time very satisfactorily (*Dieffenbachia* stem, *Allium* bulb coats, etc.). When carried through into balsam they generally lose in visibility from too close approximation to the refractive index of the medium.

Herbarium specimens or stored dried crude vegetable drugs are often called upon for histological material. The lower groups of plants are con-

sidered separately, but certain general rules apply to them and to the vascular plants in common and may be discussed here. In general the parts desired must be cut away with great caution. This is particularly the case in studying plants in public or borrowed herbaria, for from such no portion should ever be removed except for grave cause and after specific permission. The fragments, if desired for microchemical studies, must receive special treatment depending on the character of the substances to be retained. For ordinary purposes they can best be moistened with alcohol to reduce the air film, then soaked up with water; boiled if necessary to hasten the process. Lactophenol is excellent to soften specimens, as is lactoglycerin with a little water, and dilute caustic potash may sometimes be used (p. 139). Even if it is desired to macerate (p. 121) the tissues for epidermis, bast or wood fibers, idioblasts, etc., it is best to soak it up thoroughly first to assure even action of the macerating fluid. The same methods are used, after the material is soft, as are applied to material preserved in liquid, and in many cases the results are equally satisfactory. Herbarium material may also be imbedded for histological studies. Very thorough soaking should precede the dehydration, and the use of a vacuum pump to remove air is especially advantageous. The usual methods are applied (pp. 6, 126).

Inulin can readily be retained in the tissues if the material when freshly collected is cut into about 1 cm. blocks and treated with several changes of absolute alcohol, to remove the water rapidly and completely. Sections must be cut with a knife flooded with absolute alcohol. The precipitated sphaerocrystals of inulin may be observed in absolute alcohol, redissolved in water, or the dehydrated sections may be stained with orange G dissolved to saturation in clove oil, washed in xylol and mounted in balsam, the inulin being darker in color than the general tissues.

Latex-containing plants should be collected in such fashion as to prevent the loss of too much fluid before the fixation is effected. Where there are long latex vessels "bleeding" may be prevented by ligating the stem near the end of the piece to be cut away. It is preserved in 50 per cent alcohol without the removal of the ligature or clamp until hardening is complete. For further treatment see p. 135.

Resin-containing material (e. g. gymnosperms) should be immersed in a saturated aqueous copper acetate solution for a considerable period (one to several weeks) after which the excess copper may be washed away and the material preserved in 50 per cent alcohol. Sections are reported as retaining the bright green coloration of the resin when mounted in glycerin.

Sieve tubes (p. 136) containing a considerable quantity of coagulable protein substance are usually satisfactorily shown when put up in alcohol, but the distribution of coagulum within the mature tubes is not always correctly maintained. It is best to immerse the plant to be studied, or a long unsevered branch of it, for a few minutes in boiling water to coagulate the contents before cutting it up into lengths suitable for preservation.

Starch-containing material should be preserved in alcohol rather than formalin or with acids which might hydrolyze it, if the retention of the starch in good condition is desired. In most histological work it can well be dispensed with.

2. **Preserving media for plant tissues,** while offered in a wide variety of forms, are founded on comparatively few basic formulas. The safest general purpose preserving fluid is 70 per cent ethyl alcohol. To minimize loss by evaporation 5 to 20 per cent of glycerin may be added. While the strength of the solution may be reduced to 50 per cent alcohol for tough specimens destined for superficial morphological study it should be increased to 85 per cent for exceedingly delicate material or material for cytological study. Glycerin is generally to be avoided if the material is later to be imbedded in celloidin or paraffin, and if present should be removed by very careful washing before the final stages in dehydration. Filamentous algae and fungi are advantageously transferred from the wash water, after fixing or staining, to 5 or 10 per cent glycerin which is concentrated by evaporation, the concentrated glycerin serving alone as a very good preserving fluid. A general fixing and preserving fluid consists of 70 per cent alcohol 96 parts, formalin 4 parts. A recently suggested fluid contains 50 per cent ethyl alcohol 100 c.c., formalin 6.5 c.c., glacial acetic acid 2.5 c.c. It is probably better than the preceding, and is very good as a histological fixing agent, preserving the cell contents tolerably well. Formalin alone in 4 per cent aqueous solution has a general application for all tougher material intended for morphological studies and keeps it in a less brittle state than strong alcohol. It should be used with great caution on segmented Rhodophyceae, since these often disarticulate after a few months. This can be avoided in part by storing in the dark and using neutralized formalin (by the addition, according to W. R. G. Atkins, of borax until the solution gives a red color with phenolphthalein) which also will serve for the preservation of calcified organisms, although if possible these should be kept in alcohol. Preservation in Keefe's fluid (p. 139) or Stromsten's fluid (p. 158) is also effective. Methyl alcohol may frequently be substituted for ethyl when the latter is unavailable, but denatured alcohol should be avoided if possible because of the cloudiness of the aqueous mixture.

3. **Decalcification of plant tissues** must be conducted in a gentle fashion as it is of first importance to avoid distortion due to the liberation of gas or to the direct action of acid on the tissues. For morphological studies of most plants a dilute solution of hydrochloric acid (2 per cent) is suitable, but for more rapid results a 10 per cent solution may be used. The solution should be changed freely until all lime has been dissolved. For more gentle action acetic acid may be substituted; nitric acid is also used, but is rather violent.

If a more accurate preservation is desired it is well to effect decalcification by the use of one of the common fixing fluids, such as chrom-acetic, Flem-

ming, or sublimate-acetic. The solution must be used liberally, and in the case of Flemming the later stages of decalcification may be completed with chrom-acetic if the amount of osmic acid that would be consumed is an important item. If gas tends to collect in intercellular spaces it will interfere with the action of the acid, and even distort the tissues, and should be frequently removed by the use of an air pump. If the material is rather soft it may be possible to fix with formol-alcohol and decalcify after hardening in strong alcohol by the gradual addition of acetic acid.

Formaldehyde, gradually decomposing to formic acid, will eventually reduce or remove the calcification from stored specimens, so that if the deposit is not great it may soon be dissolved. This is particularly noticeable in the case of slightly calcified tropical algae, which after two to three years may be completely decalcified. For means of preventing this see p. 112. Some phycologists find Perenyi's solution (p. 422) an acceptable decalcifying agent. (See also Bone and Teeth pp. 257, 265.)

4. Desilicification of plant tissues is generally effected with hydrofluoric acid. The specimens to be treated are brought into a wax vessel or a paraffin coated bottle and flooded with acid, full strength if the specimens are heavily impregnated, or diluted to 50 per cent if they are softer. Dry woods must be boiled thoroughly and exhausted of air under an air pump before placing in the hydrofluoric acid. Capsules of mosses, etc., must be punctured with a needle to let air escape. The action may take several weeks, and the container should be kept out of doors (as on a windowsill) and not in a laboratory, since the fumes are detrimental to apparatus. A new proprietary fluid, Diaphanol, has recently met with some favor as a substitute for hydrofluoric acid. It may be used for indurated tissues in general, both lignification and chitinization being amenable as well as silicification. It is suited for use on partly carbonized peats, producing a bleaching as well as a softening effect.

5. Dehydration and clearing of plant material is to be conducted with regard to the condition of the material, bulk or section in form, and to the purpose, histological or cytological, to which it will be put. Most material of plant tissues destined for histological studies will either have been fixed in formol-alcohol or some mixture requiring little if any more attention than washing and preservation in 70 per cent alcohol. Material fixed in strong chrom-acetic may, after washing, deserve dehydration through 15, 25, 50 to 70 per cent alcohols with three to six hours in each change. For work on most root tips and on the development of the sporangial structures in phanerogams and ferns the series should be extended. If it has not been possible to actually lay bare the archesporangium it is not often worth while to extend the series unduly. For such, gradations of 10 per cent intervals serve all practical needs and the time of immersion may vary from fifteen minutes (small, uncuticularized objects as delicate root tips) to one to two hours (large ovaries, young cones, etc.). When serious cytological

work is contemplated on dermatogen cells of root tips or archesporia which have been laid bare before fixation, or on easily permeable algal or fungal material, the series can advantageously be made more gradual with a 2.5 per cent initial stage and after 5 per cent, then 5 per cent gradations to 30 per cent and 10 per cent gradations to 80 per cent. Marine organisms should sometimes have an alcohol dilution series based on sea water (p. 115). See also the smear methods for plant cytology (p. 116).

For filamentous organisms it is often advantageous after washing, simply to place them in 5 per cent glycerin, which is then concentrated by evaporation in a warm place (as above a radiator) protected from dust. When it is thick and syrupy, material can be stored in it, or for imbedding the glycerin can be washed out with 95 per cent alcohol. This method can be extended to bulkier tissues (root tips etc.), but with questionable advantage.

Dehydration can be effected from the water-saturated stage by immersion of the material in liquefied (90 per cent aqueous) carbolic acid. This is a rough treatment, but suited to the clearing of leaves etc., destined for whole, unstained mounts. Thin sections containing alcohol- or water-soluble crystals, etc., can frequently best be dehydrated simply by drying on the slide.

The dehydration of sections is almost invariably effected by passing them through a graded series of alcohols. The length of the series is determined by the critical nature of the material. For ordinary histological studies a sequence of 30, 50, 70 and 95 per cent alcohol should be quite enough, and often 50 to 95 per cent will serve. For cytological studies the sequence must be more detailed, and care must be taken that the surface fluid remaining on the slide from one change is really replaced by the higher grade, by dipping the slide several times, or long immersion. Always dehydration of sections (not in celloidin) is completed with absolute alcohol unless a clearing fluid of special character is used. In extreme cases the slides may be laid in flat dishes and dehydrated by the concentration of weak glycerin. Generally a sequence of 5, 10, 20, 30, 40, 50, 60, 70, 83 and 95 per cent ending with two or more treatments with absolute alcohol is quite correct. More wide-spaced changes are justified only when staining with aniline dyes that come away readily, and are to be looked upon with suspicion when dealing with work of anything like critical character. Carbolic acid and glycerin can also be used for dehydration purposes if occasion requires. Rehydration of intact specimens, as also of sections, can usually be accomplished by the use of fewer changes than when removing the water, as the tendency is to swell rather than to shrink the cells.

Clearing of sections of plant material is necessary between the alcohol and the mounting medium stages. A graded series of absolute alcohol-xylol mixtures is the best method, though hardly the least troublesome. For plant material the series should be almost as long as for the dehydration of the sections. After 2 to 3 changes of pure xylol the material may be placed in the resinous mounting medium. Cedar oil may be used to clear sections

from absolute alcohol, but is rather viscous and easily clouds with moisture from the air. Clove oil may be used readily after absolute alcohol and will clear from 95 per cent alcohol or even lower; it is of great service in cytological and histological work, but for the former cannot compete in delicacy with an extended alcohol-xylol series. Aniline oil may be used to clear from alcohols of as low as 90 to 95 per cent, but is hard on stains and of no value cytologically. For use with celloidin a mixture of carbolic acid (crystalline state) and xylol (about 25 per cent of carbolic, or less) is best, as it clears easily and safely from 95 per cent alcohol, and does not seem to hurt stains. Eycleshymer's fluid is sometimes ruinous to stains. After any of these clearing fluids it is necessary to wash out thoroughly with xylol, as most of them have some effect on the dyes or the mounting medium. Benzol and toluol may be substituted, if desired, for xylol in most procedures.

Clearing of plant material in bulk for paraffin imbedding is effected with the same substances that are used for sections. The best, by far, are cedar oil, and xylol in an absolute alcohol-xylol series in which the strength intervals must be closer than for sectioned material, and the periods of immersion longer; perhaps half an hour in each grade for root tips. With cedar oil it is customary to place some oil in a vial and an equal volume of absolute alcohol containing the material above it. As the objects sink through the surface of the oil they become gradually impregnated. When on the bottom the specimens should be washed for some time with fresh oil, then washed clean of it in xylol, and infiltrated. The alcohol-xylol series is much the most precise.

Dehydration of marine plant material may be somewhat complicated by the presence of dissolved salts. While it is generally easiest gradually to replace a fixing fluid with fresh water after killing marine plant tissues, it is sometimes desired to dehydrate immediately after washing with salt water, or without previous washing. In such cases the following table of fresh-saltwater mixtures will generally enable the material to be brought into 50 per cent alcohol without shrinkage or cloudiness, but intermediate stages may be interpolated for most critical studies.

Water, fresh	Water, salt	Alcohol
5 parts	90 parts	5 parts
10	80	10
20	65	15
30	50	20
35	35	30
40	20	40
50	.....	50

II. Reduction of Material to Physical Proportions within Range of Microscopial Observation

1. Non-Section Methods.

a. Smearing. The acetocarmine method as adapted to plants by Belling* is unsurpassed for immediate results in making chromosome counts and other observations of an urgent nature. The preparations secured are essentially temporary, although they may be sealed with vaseline and kept for some days or even weeks. More than one schedule is available.

The acetocarmine stock is prepared by boiling an excess of powdered carmine in 45 per cent glacial acetic acid. In a drop of this the anthers are teased out with steel needles and mounted. The chromosomes will be reddish, darkening on keeping to purple. A second schedule calls for the addition of a trace of ferric hydrate solution to the stock acetocarmine, stopping short of the production of a precipitate. Then dilute with an equal quantity of unmodified acetocarmine. Tease out the anthers with nickel instruments. The third schedule involves fixing the anthers in a mixture of 1 part of glacial acetic acid to 9 parts of absolute alcohol which has been colored brown by ferric hydrate (the amount varied to produce the best results). After days, or even weeks, the anthers are teased out in the original acetocarmine with nickel instruments.

This method has been enthusiastically adopted and with it many valuable results have been obtained. As the cytoplasm swells after a time the chromosomes can be squeezed apart from each other by pressure on the cover glass and this often facilitates counting. Belling recommends† the use of water-immersion objectives and of green light screens (Wratten filter 57a or 58) in the observation of acetocarmine preparations. How much more than the count can be effectively studied with this method is uncertain to the present writer. Certainly Belling has secured results on chromosome form in *Hyacinthus*‡ that are of a very convincing quality. However, most of the workers with this fluid do not possess equal skill and experience, and the results generally show a congeries of distorted chromosomes. The present writer has himself watched the chromosomes of *Gasteria* swell under the action of acetocarmine. Certainly he feels that it is absolutely unsafe to make any statements as to meiotic prophase conditions earlier than diakinesis based on this method. Further, statements of chromosome form must always be distrusted unless confirmed by careful fixation and staining following either the paraffin, celloidin or the balsam smear methods by their most careful schedules.

The hematoxylin-balsam smear method, yielding permanent results, was adapted to plant material by the writer some few years ago. Microspore mother-cells were so "smeared" as to cause the cells to adhere to the

* Belling, J. *Amer. Nat.*, 1921.
† *Ibid.*, 1923.
‡ *Ibid.*, 1924.

slip without any artificial cementing agent, but sufficiently strongly that they could be stained and mounted in position. Originally designed simply to facilitate chromosome counting, it became evident that the method was fitted to give results that would establish quite new standards in preparations of nuclear structures. Various types of temporary preparations have been developed (p. 116) which serve tolerably for making chromosome counts, yet none seems worthy of serious consideration in studies on the critical prophase and telophase stages or on chromosome structure. Those based on acetic acid especially are objectionable for their distortions of chromosome shape and of the spireme. While the chromosomes retain many of their structural features (constrictions, etc.) they are not usually accurately represented. The method developed by the writer retains all the best features of the invaluable chrom-osmo-acetic fixation method. It applies this fluid under the only conditions in which it can perform its best service, namely, with the important cells directly exposed to the unimpeded action of the fixing solution, and with almost instant contact of cells and solution. The cells, after fixation, can be washed, stained, dehydrated and mounted in balsam by Heidenhain's hematoxylin technique, the most accurate devised for nuclear studies. Shrinkage of the protoplast is enormously reduced (often apparently eliminated) and this is not merely due to the unbalanced swelling action of acetic acid. In addition to the orthodox technique this method offers by far the most crucial opportunity for testing other fixing fluids and their variants or adjuvants, because, since the cells studied are directly exposed to the fluid there is no question as to the filtering action of superposed cell layers (anther walls, etc.). The precision of response to changes in the fluids is much greater by this method than by any other. By it the writer and his students have been able to secure most valuable data on the structure of the spireme, to demonstrate the chromosome tetrad in the late prophases and the structure of the chromosomes, to determine most accurately the constrictions related to the spindle fiber attachment, and to reduce or even eliminate the contraction feature which was supposed to be an essential element of the synizesis (synapsis) stage, provided the material is in such a state as to permit smearing at this meiotic period. The chromosomes show far less tendency to clump than in imbedded and sectioned material. The cells can be scraped loose from the slide and caused (by local pressure) to rotate in the balsam under observation so that all views may be seen of a single meiotic or mitotic figure. The mounts are, of course, permanent records, which is not the case in the glycerin or acetic methods. As was noted when the process was originally described:

"Because the cells are spread out in a single layer and in immediate contact with the fixing fluid, the quality of the fixation may be very high. The karyolymph is precipitated in a uniform and delicate way, giving the much desired 'solid fixation' of the nucleus, as a result of which the chromatin elements retain their normal position within the

membrane, resisting the tendency to clump at various stages so familiar in paraffin material. The cells are entire, and the observer may work without the necessity of accounting for parts of one cell displaced in two or three sections. This is not always an advantage, but in making chromosome counts it is much to be desired, and this method gives entire metaphase plates without broken or cut chromosomes. It might be thought that the making of the smear would rupture the pollen mother-cells, or at least displace the contents within them, but it is found that most of the cells escape damage, and in the others it shows so obviously that there is almost no possibility of confusing normal and abnormal conditions."

The method has been used in extended studies on *Gasteria* and *Veltbemia* by the writer, and by Kaufmann (p. 119) on *Tradescantia* (*Rhoeo*) and *Podophyllum*. It has also been used on several other forms, such as *Haworthia* and *Pitcairnea*.

The important limitations of the method are, first, that it can be applied only to cells not joined together by a firm middle lamella. In higher plants this means, essentially, spore or microspore mother-cells after they have begun to round up (the beginning of the breakdown of the archesporium) although occasionally a fairly intact archesporium may smear tolerably well. Secondly, the thick jelly-like wall around the microspore mother-cells and the quartets (tetrads) interferes with the use of aniline dyes as counterstains, although safranin and some others can be used as satisfactory single stains. It does not succeed well on species which are heavily gorged with stainable food material during the maturation divisions, or on pollen grains after the coagulable material in the anther sacs has disappeared.

The method was originally described in part as follows:

"The fixing fluid used is a chrom-osmic-acetic mixture of the following constitution: 10 per cent acetic acid 2 c.c.; 10 per cent chromic acid 0.2 c.c.; osmic acid dissolved to 2 per cent strength in 2 per cent chromic acid 1.5 c.c.; distilled water 8.3 c.c. About 1 per cent of maltose has usually been added to this fluid, which will probably have to be modified to suit each plant on which it is used. A slender glass rod is laid in the bottom of a petri dish, and enough of the fixing fluid poured in to cover it. It has been found more convenient to use slides than cover glasses, and these are cleaned by long immersion in battery fluid, rinsed, and dried with an absolutely clean cloth. The anthers are excised, and as soon as collected are crushed and spread over the center of the slide with quick strokes of a clean scalpel, which must be honed flat and smooth on the face or the smear will not be a success. The slide must be immediately inverted on the fixing fluid, bringing it down in a horizontal position, so that the whole smeared face is wet simultaneously. The time from the first crushing of the anther to fixing should not exceed three to five seconds. If the slide is brought down on the fluid in an oblique position much of the material will wash off. The slide may stay in the Petri dish, resting on the glass rod for a few minutes, while a second is prepared and placed beside it; then it may be removed, turned right side up, flooded with the fluid, and left for the full time of fixing, or about fifteen minutes. The slides are then to be washed in changes of water in staining wells for about an hour, and any large pieces of anther walls, filaments, etc., which have not broken loose, can be picked off with a needle. A preliminary inspection of the material also can be made, and slides showing stages which are not desired may be discarded. The developing pollen mother-cells adhere excellently, and rough rinsing does not dislodge them. Smears of pollen grains are quite easily made, so long as they are still

surrounded by viscous fluid in the anther, and a very fair fixation may be secured through the chitinous wall, so that the first mitosis within the pollen grain may be critically studied.

"If the slides are to be stained at once, they are then bleached in diluted aqueous hydrogen peroxide, rinsed again, and placed in 2 per cent iron ammonia alum solution; or they may be partly dehydrated and held in 70 per cent alcohol for later staining. After four to twelve hours in the iron alum they are again washed most carefully for fifteen minutes or more in running water, and stained for four to twelve hours in 0.5 per cent aqueous haematoxylin solution, followed by another rinsing, destaining under observation in the iron alum, and an hour of final washing. Dehydration is to be accomplished gradually through alcohols by 10 per cent stages, but the slides need remain in each stage only two to three minutes. Clearing must be done by mixtures of xylol and absolute alcohol, of which about four intermediate stages seem to be enough. In this connection it must be noted that the somewhat mucilaginous walls of the developing pollen mother-cells are intact, and as a result any great change in the density of the successive solutions into which the slides are introduced is sure to cause collapse of the walls and shrinkage of the protoplast. After the smears have come from pure xylol, very dilute xylol balsam is dropped on them and allowed to concentrate by drying for a few minutes before the cover glasses are put in place.

"The chief difficulty encountered is that of getting a brilliant stain, but the writer has eventually succeeded in doing so in those plants which he has so far tried. The haematoxylin and iron alum must both be of the best grade and in good condition, but in spite of care some batches of slides will show a muddy stain. Passable results can be secured within a single working day by shortening the staining periods, thus enabling chromosome count work to be done very rapidly, with the advantage over the acetocarmine method of permanency of the slide records." McClung* has published critical studies of fixative action on smears of animal tissue.

Working in the present writer's laboratory, Kaufmann has studied the adaptation of this smear method to a variety of materials, and has recently offered a very valuable modification of it. His results demonstrate a very remarkably consistent spiral structure in meta- and anaphase plant chromosomes and enable a fairly continuous interpretation of the mitotic cycle to be presented. He suggests the following plan:†

"The present writer [Kaufmann] encountered the same trouble [occasional muddy stain] in early efforts to secure consistently good results, but the following alterations in the staining schedule increased materially the proportion of usable slides. Instead of mordanting in 2 per cent iron alum for a period of four to twelve hours, as suggested by Taylor, the same concentration was used for forty-five minutes to one hour. Washing in running water followed for about ten to fifteen minutes. The staining process was curtailed to twenty or thirty minutes in a 0.25 to 0.50 per cent solution. The necessary time can be estimated after a few trial experiments by the color concentration in the cells. As soon as a deep purplish-black appeared it was found advisable to transfer the slides to water. Differentiation in the 0.50 per cent solution of ammonioferric alum then could more often be completed prior to the appearance of a muddy color than was possible with the prolonged staining.

"The iron alum was diluted as desired from a 20 per cent stock solution. The haematoxylin was of American manufacture and certified by the Commission on the Standardization of Biological Stains. No advantage seemed to accrue by allowing the solution

* McClung, C. E. *Anat. Record*, 1918, xiv, 265.

† Kaufmann, B. P. *Stain Technology*, 1927.

to 'ripen' for any considerable time. Often the crystals were dissolved not more than one hour prior to the time of staining. The solutions were used but once or twice as a further caution against the muddy color.

" . . . The curtailed periods of staining facilitate the handling of a considerable number of slides in the course of a day. If a suitable plant is available which shows active cell division during the morning hours, it is possible to smear 40 or more slides at that time and to present the permanent mounts by evening. The writer has found a flat honed scalpel with a straight cutting edge the most advantageous for making smears. Small anthers like those of *Tradescantia* and *Rhoeo* can be crushed and the contents spread with one stroke of the scalpel. While spreading the cells an effort is made to increase gradually the pressure on the scalpel. In this way some cells will usually be found deposited in but a single layer, although others may be crushed. Some excellent preparations of chromosome structure have been obtained at that intermediate zone where the pressure on the scalpel was great enough to rupture the cell wall but not sufficient to bestroy the protoplast. Success in preservation is due evidently in these cases to the instantaneous penetration of the fixative.

" . . . Of the various fixatives employed the chrom-osmic-acetic acid and the picric-acetic-formaldehyde combinations proved the best. In most cases penetration was enhanced by the addition of such adjuvants as lactose, maltose and urea in concentrations ranging from 1 to 5 per cent. It is impossible, however, to detail a single formula which proved adaptable to all phases of meiosis. For example, Bouin's fluid plus maltose was excellent for preserving the later prophase stages of the first maturation division but was not so adaptable to other phases."

Very recently Newton* has recommended the use of gentian violet as a stain, followed by dehydration in alcohols containing 1 gm. per cent each of iodine and potassium iodide, whereby it is possible to obtain stains of any intensity required, while the transparency of the cytoplasm renders easy the examination of thick sections or smears. As Newton's results are of the utmost significance in demonstrating a tetrad structure in meiotic prophases his staining method deserves careful trial. The gentian- or crystal-violet stain as applied to a fixed smear may be superbly differentiated, as shown by an example (*Tulipa*) kindly sent to the present writer, who has in confirmation also secured very fine differentiation on root tip (*Allium, Fritillaria*) material. If destaining with iodine solution is too slow it may be alternated with clear alcohol. Observation of such slides is further facilitated if a Wratten B green screen is used below the microscope condenser, whereby the stain appears almost as black, clean-cut and sharp as in a hematoxylin preparation, or they may be used with a Wratten K_3 yellow screen, when the stain appears a deep, sharp and rich red.

Since the procedures outlined here are rather new to botanists they have been given in detail in order that no lack of reasonable suggestions should prevent their wide and careful trial. They do not relieve the technician from the necessity of intensive effort in mastering the method. For its finer results this smear method calls for a building up of personal skill quite equal to that required for mastering the paraffin method, and must be attained in part independently of a knowledge of that method. Zoologists have used smear

* Newton, W. C. F. *Linn. Soc. J. Bot.*, 1927.

methods effectively in many of their most critical studies for many years, and a real advantage is to be gained by their adaptation to plant cytology.

b. Teasing methods are excellently adapted to many botanical investigations. Much easily disorganized plant material is readily separated into its constituents by patient work with fine dissecting needles. The motion used should be a stroking or combing one, following the direction of the fibers, if of filamentous or fibrous nature. The general operation is similar to that used on animal material (p. 5). It is frequently of advantage to give a brief maceration before attempting to tease material apart. Extremely delicate filamentous algae or fungi are generally teased after partial dehydration and hardening. Rarely is it of advantage to tease material after clearing or infiltration with balsam, but the method may be employed when the products are too minute to be readily retained otherwise (asci of *Peziza*, wood fibers in small quantity).

c. Maceration of plant epidermis to facilitate its removal for studies of hair or stomata may sometimes be effected by mechanically stripping the slightly wilted leaf or stem. In most cases, however, the mesophyll (or cortex) tends to adhere and must be loosened before stripping. For the most delicate leaves scalding or boiling may suffice. More resistant cases usually respond to boiling in a solution of KOH (5 per cent, stronger or weaker as results indicate) until the tissues become translucent and tests show that the epidermis comes away most readily. A very careful brushing of the inner surface will generally clear away mesophyll debris. In some cases (*Sarracenia*) the epidermis cannot be stripped, but the epidermis of the partly macerated leaf must be scraped free from subjacent tissue with a scalpel, or the HNO_3-$KClO_3$ method (below) may be adapted with favorable results. After the maceration fluid has been washed out of the material it may be stained with eosin and mounted in 2 per cent acetic acid or glycerin, or mounted in glycerin jelly, or stained with safranin or other suitable dye, dehydrated and cleared for mounting in balsam.

d. The maceration of woods to yield wood fibers is an important method because the study of the individual types of tracheal elements in vascular tissues is best effected with isolated fibers. Longitudinal sections permit but a single view of a given fiber and rarely all of it, while suitably macerated fibers show in their full extent. Bundles from an herbaceous fern (such as *Pteris*, which responds very well) or monocotyledons are best separated by hand from the surrounding soft tissue and are cut up into convenient lengths (2 to 10 mm.). Wood from perennial arborescent types (as for instance *Tilia*) is reduced to very thin longitudinal chips or shavings. The material is then gently boiled in 50 per cent HNO_3 with the frequent addition of crystals of $KClO_3$. It is well to stop treatment when the pieces of material begin to fray out at the ends, but the exact point must be experimentally determined for the particular subject. Wash with several changes of water by settling and decanting, and examine. Boil in safranin 0 or

other strong aniline dye, wash and dehydrate very quickly with 95 per cent alcohol (2 or more changes), absolute alcohol and xylol, allowing the material to settle and decanting the changes. Nigrosin (saturated) in saturated aqueous picric acid is also a useful stain. While dehydration must be thorough, decolorization is rapid and dehydration must be rushed as much as possible. The addition of a small portion of xylol to the second and subsequent alcohols will reduce the loss of the stain. Material cleared without staining may sometimes be satisfactorily stained in xylol by adding a few drops of a clove oil solution of the dye (as of light green). Maceration of woody tissues may also be effected with very strong hot aqueous chromic acid (30 per cent or stronger). This method is also used to demonstrate the laminated wall structure of *Tribonema* (Heterokontae) and other algae.

2. Section Methods.

a. Grinding. The preparation of fossil plant material for microscopic study does not offer much variety of method. Something may be learned of the structure of semifossilized peats or of soft brown coals by macerating and bleaching small pieces with Diaphanol, nitric acid, or aqua regia, with subsequent washing and teasing out of the tracheids, spores or pollen grains. Or the samples may be desilicified with hydrofluoric acid and sectioned in the sliding microtome with or without imbedding in celloidin under pressure. Jeffery* recommends that the material be soaked in carbolic acid under pressure in a wired stoppered bottle in a paraffin oven both before and after the hydrofluoric acid treatment, and the repetition of the treatments with both chemicals, if needed. The periods of immersion in each should be about a week. Staining is not usually practicable, and bleaching (p. 130) is often indicated. Sometimes fragments of tracheids or epidermis can be removed from fossil impressions of leaves or twigs by aid of Diaphanol or nitric acid, but generally microscopical observations are effective only on material infiltrated with silica, etc., without destruction of internal tissues. Harder bituminous coals and fossil woods, seeds, etc., are generally sectioned by grinding. With a hack-saw and a blade of the hardest temper (for cutting steel, etc.) thin slabs should be cut in the desired plane through the specimen. For detailed directions regarding grinding sections see p. 265. Radial (and often tangenital) slabs can frequently be split from fossil woods with a cold chisel. After the specimen has been ground as thin as possible while held in the hand, one surface is polished. A heavy glass microscope slip or a piece of plate glass is to be ready and clean, and a polished surface of the specimen is cemented to this with the resin-lanolin cement used hot (p. 140). If the cement is too flexible it may be of advantage to reduce the amount of lanolin. As soon as firm and cool the final grinding may begin. After grinding and final washing and drying with alcohol the specimen is to be very carefully slipped off the grinding slip, well cleaned with xylol and mounted with thick warm balsam, weighted

* Jeffery, E. C. Anatomy of Woody Plants.

to squeeze out excess fluid and allowed to harden. As the specimen approaches desired thinness the danger of its breaking up increases enormously, and great care is required, especially to see that the specimen is of equal thickness throughout, and that the two surfaces are therefore parallel. This is particularly hard with dense black material. Some especially friable specimens have to be saturated with resin before grinding, and cannot be freed from it or the grinding slide before mounting for fear of breaking to pieces. All that can be done is to give a careful washing with water, perhaps a dip through alcohol, finally drying and covering with balsam and a coverglass.

b. Cutting. Free-hand sectioning of plant tissues gives the opportunity for answering so many of the simpler questions which arise regarding structural conditions in larger plants that all students should be compelled to acquire a considerable facility in the process. For the cruder examinations a safety razor blade held in the fingers or in a flat holder will serve, but for the exercise of skill in producing sections worthy of preservation a regular heavy sectioning razor should be employed. This must be ground absolutely flat (or at most imperceptibly concave) on the back, and honed flat with the imperative avoidance of a secondary bevel near the edge. No "honing back" such as is used on paraffin knives can be tolerated here. The upper face should be ground concave, and held evenly on the hone in sharpening.

The material to be cut is placed between two split pieces of elder pith (p. 125) and held in the left hand supported against the thumb by the forefinger. The razor is held as in shaving by the right hand and rested on the bent forefinger as it is drawn in long sweeping strokes from heel to tip across the specimen. If the arms are resting on the table or against the body there is no likelihood of cutting the supporting thumb even when this is held in the correct fashion much higher than the specimen. The sections obtained will not be as continuous or as uniform as a microtome delivers, but they will reflect in quality the care of the technician, and the speed with which a specimen can be sampled will often more than compensate for a slight loss in quality.

Hand Microtome Sectioning of Plant Tissues. Intermediate in convenience between free-hand sectioning and the use of the large sliding microtomes come the hand microtomes.

The essential feature of these is that the knife is not attached to the instrument, but is held in the hand and slid across the glass top of the instrument. It may be a chisel shaped knife or a hand razor, and in the latter case must be held firmly flat on its back upon the instrument. The specimen is held in a clamp and this is pushed toward the top by a screw at the bottom of the tube, the drum head of the screw being graduated to indicate the degree to which the specimen is raised by the parts of each revolution. An emergency instrument of this type is afforded by a spool of appropriate size, through the hole of which the specimen may be pushed and held by one hand while the razor is drawn across the end with the other.

Sliding microtome cutting gives the most exact method of preparing sections of uninfiltrated plant material. While reconnaissance observations

of root, stem or leaf anatomy will usually be most quickly and satisfactorily performed upon free-hand sections, the material for final staining and preservation as research data will best be made with an accurate sliding or sledge microtome. Either living or preserved plant material may be sectioned without imbedding provided it is sturdy enough to withstand clamping in the carrier. For the harder woods, seeds etc. see p. 122. Soft tissues may be imbedded, as in celloidin (p. 126) or paraffin (p. 126), or frozen (p. 125). Stems and roots of ordinary hardness (*Pelargonium*, *Helianthus*) are cut to appropriate lengths and placed between the jaws of the clamp. It is well to protect the material by placing it between split pieces of elder or sunflower pith (p. 125), or even better between pieces of carrot which have been kept fresh and turgid by soaking in water. The knife should be set in as oblique a position as practicable so as to give a long slanting stroke, but for hard tissues the knife may bend too much in this position if it has not a clamp at both ends, in which case only the edge toward the heel can be used. The knife and specimen should always be kept flooded with water, or with alcohol of 50 to 70 per cent on preserved or soft material, and the sections removed with the finger or, if great caution is exercised, with a camel's hair brush. Sections may be cut at 10 μ to 20 μ if they are very favorably adapted to the method (*Populus*, *Tilia*) but spongy collenchymatous tissue (*Dieffenbachia*) may have to be cut as thick as 100 μ or more in some cases. Leaves may be cut in bundles, or rolled up, many sections resulting at each stroke of the knife. Very slender roots, moss stems, setae, etc. may be cut in bundles also, and through the later stages handled with a pipette. Softer woods should give no trouble for sections in any plane, but when harder they may have to be boiled for a time to soften them or treated as on p. 127. Generally on this material it is necessary to hone the knife after every few sections are cut. In this way it may be possible to avoid a wire edge. Silicified or carbonized material must be treated with hydrofluoric acid as on p. 113.

Rotary Microtomes. These are used for cutting sections of plant material imbedded in paraffin in exactly the same way that they are used for animal material (p. 17). As it is often necessary to sample large quantities of material before deciding which specimens are to be finally completely reduced to sections and preserved, botanists prefer to leave the specimen attached to the block which holds it in the microtome until the test slides have come through the staining processes. This often necessitates a very large number of supporting blocks. These can most economically be prepared by cutting a $\frac{3}{8}$ inch board of white pine transversely into strips 1 inch wide. These can be quickly split into the desired sizes with a heavy scalpel. One end of each block should be dipped into hot paraffin before mounting any specimens upon it, in order that the pores may become filled with paraffin and the specimen enabled to stick better. One inch lengths cut from "dowel" rods of appropriate diameter are very convenient.

Pith for Protecting Plant Tissues. Used when sectioning without infiltrating. Pith is usually derived from the elder or sunflower. It is less liable to become soggy when sections are cut if it is soaked and kept until used in an equal mixture of 95 per cent alcohol and glycerin. For many purposes fresh root of carrot is preferable.

c. Imbedded Material.

The Freezing Method of Imbedding. This has little general use for plant material. It is, however, important in dealing with fragile pathological specimens and with soft or gelatinous algae. The usual method should be followed (p. 29). Fixed material should generally be brought to formaldehyde (about 4 per cent) and specimens should be coated with egg albumen, gelatin or gum-arabic and attached by it to the carrier. The sections may be handled in syracuse watch glasses or laid upon slips coated with gelatin and cooled. The slides are later warmed to cause the sections to adhere and then treated as in the paraffin method (p. 129), avoiding dyes which might persist in the gelatin.

The Gelatin Method of Imbedding. This is frequently of advantage when there is occasion to give special support to fragile plant tissue which is liable to crumble and which becomes brittle in celloidin, such as diseased bark and woods. Gelatin is allowed to absorb as much water as possible, drained, melted, and the material, cut into as small blocks as practicable, is soaked in this solution. After some hours it is oriented on wooden blocks, the gelatin allowed to cool, and toughened in 10 to 20 per cent formaldehyde. The material should be cut with the knife flooded with water. If the sections are sufficiently strong the gelatin may be removed with hot water, without or with ammonia, and they can then be stained in the usual ways. Material that can not be parted safely from the gelatin will not stain very satisfactorily, but can be nicely mounted in glycerin jelly if care is exercised not to have the mountant too hot.

The Soap Method of Imbedding Plant Tissues. This has been little used, but as advocated by Osterhout for algae, offers advantages applicable to many kinds of mucilaginous or fragile material that cannot safely be dehydrated. He saponifies 70 c.c. of hot cocoanut oil with 38.5 c.c. of 28 per cent aqueous KOH. When firm the product is pulverized. The algae are placed in warm water to which the soap is gradually added until quite concentrated, when by drying it becomes firm enough to attach to a wooden block in the sliding microtome and cut (p. 123). Sections are to be placed on albumen-smeared slides, moistened with xylol and pressed into contact. The soap may then be dissolved away and the slide warmed to coagulate the albumen. Immersion in 95 per cent alcohol should serve the same purpose. The sections may be stained as after paraffin so far as their texture and thickness permit, or the sections may be mounted in water, warmed and examined at once.

The Paraffin Method of Infiltration and Imbedding. This process is used in common by botanists and zoologists with only minor differences (p. 6). Plant material, however cleared, is best passed through xylol (2 or more changes) and is then ready for infiltration. This process is subject to the delay caused by the presence of cellulose walls, so that more time is needed than when handling most animal tissue.

If fairly deep dishes (1 cm. or over) are used it is convenient to pour melted paraffin (48° to 56°c.) into them until half full and allow it to harden. The specimens are then placed upon it with enough xylol to cover them. The xylol will gradually dissolve the paraffin and when this is complete or when the mass has reached a soft pasty consistency the dish and its contents should be placed in the paraffin oven at a temperature of about 60° to 62°c. for one-half to one hour, after which the mixture is replaced with melted soft (48° to 56°c.) paraffin at the oven temperature. At the end of one-half hour this may be replaced with a mixture of melted soft and hard (56° to 62°c.) paraffin for another half hour and a second portion of the mixture substituted. This may be left for one-half to one hour at the end of which the material may be imbedded or cast into a hard block. This may be done in thin glass dishes rubbed inside with glycerin, or better in boats folded of good tough paper. The dishes should be chilled and the boats floated in cold water as soon as the specimens have been neatly arranged with warmed needles or forceps.

The data as given are open to modification in several respects. The times of infiltration are to be greatly reduced for filamentous algae, fungi or slender root tips, etc. They are approximately correct for the maximum size of block of phanerogam tissue that should be imbedded for cytological work, i. e., about 2 mm. *They should be reduced whenever possible.* If large blocks of tissue are to be imbedded for histological work the time may have to be greatly increased, but the cell contents will suffer from this exposure to heat. The paraffin should be used of as low a melting point as possible and kept just above that point in the oven. (For a simple and convenient method of keeping paraffin melted see p. 13.) If cutting can be done in a cool room or with a chilled knife a softer paraffin can be substituted with advantage to the material, but if it is necessary to work in the summer an even greater proportion of hard paraffin must be used. The method of substituting paraffin for xylol as given is that which most pleases the present writer. Some workers suggest dropping shavings of paraffin into the vial above the specimens, but this may tend to crush them if delicate. Others suspend a wire gauze basket filled with paraffin in the upper portion of the vial containing the specimens.

The Collodion or Celloidin Method. This is used for plants and animals in a very similar fashion (p. 27). The time of immersion must be greatly increased for much plant material, and the sectioning of hard specimens offers considerable difficulty. It is usually best to place the material, saturated with ether-alcohol, in a very dilute celloidin solution, stopper the bottle loosely and allow the solution to concentrate gradually by evaporation. Root tips and highly porous stems will usually be in suitable condition if the concentration is completed in from three to four days to a week, but

tissues with strong cellulose walls, or large blocks of wood, should be more closely stoppered and it may take a month or more for them to be properly saturated, and the solution must concentrate very slowly so as to be of equal density throughout the specimen. If they are sufficiently rigid to stand it (as in woods) the process can be made more rapid by using the solution in 3 strengths of about 2, 4 and 8 per cent. The weakest is first placed on the specimens in a strong bottle and the cork (which must be of the best quality) wired in strongly. Then this container is placed in a paraffin oven at 50° to 60°C. for a day or more as seems to be required. The same process is repeated for the stronger solutions. In any event the material is finally saturated with a barely fluid solution of celloidin. Supporting blocks of fiber or white pine are saturated with ether-alcohol and the end grain dipped in medium strength celloidin solution. They are up-ended and the specimens placed on the soaked surfaces. As soon as the celloidin becomes surface-firm in the air the specimens and the supporting block ends are dipped in strong celloidin, and by alternate drying and dipping a sufficient layer is built up around each of the specimens to support and hold it to the block. Then all are dropped into chloroform until quite firm (twenty-four to forty-eight hours), transferred to 95 per cent alcohol and glycerin (equal parts) until transparent, and may remain there until used. The sections should be cut with a knife flooded with 70 per cent alcohol and placed in a very oblique position. For general remarks on staining see page 133. For plants Heidenhain's hematoxylin followed, if desired, by erythrosin, or safranin O, followed by Delafield's hematoxylin, give the most satisfactory results. Most aniline dyes are unsatisfactory because they stain the celloidin deeply. If this can safely be removed the range of dyes available suffers no special limitations from the imbedding, and ether-alcohol, clove oil and absolute alcohol all will remove the celloidin if the sections are sufficiently tough to remain intact in its absence. Plant material is stained and dehydrated to 95 per cent alcohol in the usual way, and can then be cleared by transfer to carbol-xylol, which the writer prefers to the mixtures of essential oils. After washing with pure xylol the material is mounted in balsam. The celloidin method is generally used with desilicified materials and woods softened with hydrofluoric acid (p. 126). It can readily be so conducted as to permit the consecutive arrangement of serial sections (p. 28). For very hard material advantages are sometimes found in the double infiltration method, whereby a celloidin block is infiltrated with paraffin and cut dry, superior support being claimed for the specimens. It is probable that serious attempts to employ celloidin for cytological studies will produce results superior to the paraffin method in reduction of chromosome clumping or retraction of satellites, etc., but the stages must be most gradual and the sections very thin.

The Sectioning of Woods and Other Hard Objects Not Adapted to Grinding. This requires either extremely powerful apparatus or some method of

softening the tissues. The study of woods offers two methods of attack, one of more or less macroscopic observation, the other histological. Since distinctions between types of woods can often be made at no or very little magnification, and since these can be facilitated if thin slices be used, it has become customary to prepare large sections and to use these as hand specimens* or if sufficiently thin to use them for lantern projection. While special machines for cutting these slices have been made, they are rarely available. Remarkably good work can be done with a heavy steel carpenter's plane provided the blade is of hard temper and skillfully sharpened to a keen straight edge. Transverse sections made with this may be 1 to 2 inches broad and up to 6 to 8 inches long, and if cut from radial slabs from a tree may show cork, phloem, xylem and pith in fine condition. The sections can be cut thin enough for low power microprojection (5 to 10 cm. focal length lens). While such woods as pine and cedar cut most easily, *Wistaria*, *Vitis*, *Acer*, *Pyrus*, *Castanea* and even *Quercus* are perfectly practicable. The larger sections and harder woods make much the greatest demands on the skill of the cutter. Sections should be kept wet and may be stained (safranin, Heidenhain's hematoxylin, etc.) in petri dishes or other suitable containers, dehydrated and mounted between thin sheets of glass such as lantern slide covers. The mounting balsam should be very thick, used preferably quite warm, and the mounts should be kept warm (as in a paraffin oven) until quite hard. This is especially urgent if the mounts are to be exposed to the heat of a lantern. It assists in mounting to have one of the mounting glasses a little smaller (5 mm.) than the other, and it may be necessary to use a weight to hold the specimen flat if it is not very thin.

For accurate microscopical study of cell types and arrangements this method will not suffice. Soft woods or the alburnem of harder ones, especially in fresh material, can frequently be cut in a sturdy microtome without special preparation (p. 124). Seeds that are hard because of the presence of reserve cellulose can likewise best be cut fresh before they have become entirely mature to dryness. If dry they may become soft enough after boiling in water. Sections cut deliberately thin are more easily obtained than thicker ones. Very hard seeds may be treated with hydrofluoric acid or after the fashion of fossil woods (p. 122). Harder woods should be softened as much as possible and imbedded. As the hardness is often due in large part to the deposit of mineral matter (especially silicates) in addition to lignification it is best to remove the mineral matter before imbedding. This is effected through immersion in hydrofluoric acid which has little effect on the middle lamella and even leaves the coarser cell contents intact. Dry woods should be very thoroughly freed from air by alternate open boiling and exhaustion under an air pump, and in fact this is usually necessary with fresh woods. Pieces as small as the requirements permit (preferably 1 cm. or under) are placed in full strength hydrofluoric

* Hough, R. B. The American Woods, Ed. 2, N. Y., 1893–1913.

acid in a wax or wax-coated container and allowed to soak for one to several weeks, in the latter case changing the solution occasionally. Comparatively soft woods may be given the reduced period, or even soaked in once used or diluted acid. After the maximum softening has been attained, as determined by testing the blocks with a knife, the acid must be very thoroughly washed out. Imbedding is usually in celloidin, and if the material is porous and homogeneous by the rapid method (p. 127). The knife used on woody material should be particularly heavy and rigid and may be placed obliquely or in a more transverse position if that gives better results. It will generally need honing after every few sections, or in extreme cases every section, to avoid a turned or wire edge. The sections generally will not withstand removal of the celloidin and must be stained accordingly.

d. Affixing Methods. When handling paraffin sections, or celloidin sections in serial order, it is usually necessary to attach the sections to microscopical slips and by changing the glass with its attached sections to perform the remaining manipulations with a minimum of effort.

For paraffin sections it generally suffices to rub an exceedingly thin film of Mayer's albumen (p. 475) on the slip, following with a half-pipette full of distilled water on which the sections are floated and spread by gentle heat, the excess water being then drained off and the sections accurately arranged. It is generally necessary to maintain the sequence of the sections through the entire series, mounted on a slip, and, in order that a mechanical stage may be effectively used in recording observations, to keep the rows straight. For sections that tend to expand and contract considerably during dehydration and hydration it may be necessary to use a mixture containing gum arabic or glue 1 gm., potassium bichromate 1 gm., water 98 c.c. (Land). The slide may be flooded with this, the sections spread and the slide drained. Exposure to daylight will render the gum insoluble in the ordinary histological reagents. The use of a dilute solution of albumen instead of rubbing the slip with a minimum quantity of the strong preparation has not as wide acceptance among botanists as among zoologists, but it will work with such cytological material as has densely protoplasm-filled cells.

For celloidin sections the methods used by zoologists (p. 27) are adequate.

III. Preparation and Staining of Sections

A general preparatory treatment is required of most sections of plant material previous to staining. Separate paragraphs have been prepared dealing with problems specially related to individual major groups of plants and detailed information as to methods applicable to them should also be sought there. Others have been prepared treating of the various imbedding, sectioning, macerating, staining and mounting methods, as well as methods pertinent to special tissues. Sections cut free-hand or with

the sliding microtome need little treatment preliminary to staining. If they are highly mucilaginous it is generally necessary to remove this substance before staining by boiling in a relatively large volume of water (or several changes) until the mucilage has been completely dispersed. Otherwise it will be difficult to differentiate the stains, to prevent the sections from curling and to dehydrate them properly. Starch is frequently objectionable as obscuring cell arrangement, and can be removed by prolonged boiling of the sections in water. As the starch paste formed at first in the cells is as bad or worse than the grains the washing should be complete and the sections should be tested with aqueous iodine until the starch reaction is negative. It may be necessary to add a few drops of hydrochloric acid to the water to facilitate removal of the starch. A somewhat prolonged treatment of the sections with diastase may also be used to remove starch. Chlorophyll may be objectionably prominent in chloroplasts and although it usually disappears in the dehydration it may be better to soak the sections for a time in 95 per cent alcohol before staining.

Bleaching is necessary if plant tissues are naturally colored, or develop pigments in the cells as a result of the method of preservation, before stains may be applied. Discoloration due to osmic acid is readily removed from sections in commercial hydrogen peroxide diluted three-fourths with water, or one-half with 95 per cent alcohol, which latter solution usually keeps for several days. The bleaching is hastened by exposure of the material to sunlight. Free chlorine may also be used. A few drops of hydrochloric acid are dropped on a crystal of potassium chlorate, and when gas is formed 50 per cent alcohol is poured on it. The specimens are placed in this alcohol from 70 per cent and bleach in one-fourth hour or longer. The method can be used with sections attached to a slip. Immersion in freshly prepared Javelle water is often very effective. Sulphurous acid saturated in alcohol may be used to bleach, or potassium permanganate in very dilute solution followed by oxalic acid and exposure to light. Leaves (especially *Drosera*) which blacken when thrown into alcohol are advantageously immersed when collected in a rather strong NaOH solution at room temperature until they become bleached and translucent. After washing they may be preserved in alcohol and remain translucent and undarkened.

Staining methods for plants have been developed along several lines, of which the more general are outlined here. Staining of plants in toto has been almost discarded for the grosser types, although for unicells and for filamentous types it still serves a good purpose. For details see paragraphs on algae, bacteria, fungi, etc. (p. 164). Staining of vascular systems in pieces of stem or leaf to trace the course of the bundles may be accomplished with the living plant, killing it afterward (p. 140, ¶ 3).

Staining methods for histological or for cytological features employ solutions prepared in similar fashion. The use in botanical practice of mixtures of dyes, especially such as involve chemical action and unstable

compounds in the solution, has largely passed away. This enables a few general directions to be given for the preparation of standard stock solutions. Special staining methods will be considered separately. (For a more complete discussion of the properties of biological dyes see p. 435.)

Aqueous dyes are usually made up as 1 to 2 per cent solutions. Since the proportion of dye substance in the powder is not constant it would be better to prepare them as saturated solutions, which probably would not affect the practice greatly, as most of the dyes are soluble to approximately 1 to 2 per cent of actual substance. Among the common dyes frequently used in aqueous solutions are: aniline blue (for sieve tubes), methylene blue, gentian and crystal violets, eosin, carmine, hematoxylin and picric acid. Distilled water should always be used. The anilines all function well as 1 per cent solutions. The aqueous hematoxylin is the simple solution for use in the various separate-mordant methods. The dyestuff should be of pale brown crystals and of assured staining quality, for many samples differentiate poorly. The stock solution is of 1 per cent strength, and in use it is diluted to $\frac{1}{2}$ to $\frac{1}{4}$ per cent (histology, most cytology) or $\frac{1}{10}$ per cent to $\frac{1}{20}$ per cent (filamentous algae, etc.). The stock may be prepared by gradual solution in the cold, or by the aid of heat, but the latter had best be avoided for critical work, and preliminary solution of the hematoxylin in alcohol must absolutely be omitted, for the resulting solution behaves in a quite inferior fashion.

Alcoholic solutions are, like the aqueous ones, generally 1 per cent strength. Saturated solutions would be more constant in composition, but where used for counterstain should be somewhat diluted before use. Common dyes are generally made up in 70 per cent alcohol and include the following: acid fuchsin, light, methyl and iodine greens, bismark brown, crystal and gentian violets (for histological work), alcoholic eosin and erythrosin, auramine, magdala red. The writer uses "aniline water" (a saturated aqueous solution of aniline oil, prepared by shaking and filtering) in making up the gentian and crystal violet solutions, but not the others. Solutions with aniline oil do not always keep as well as without it, but they generally stain more densely. Aniline blue, when used for filamentous or unicellular organisms that are inconvenient to dehydrate after staining, is usually made up in 90 per cent alcohol. Safranin O, the most commonly used aniline in histological researches, is best prepared as a 1 per cent solution in 50 per cent alcohol made with aniline water (see above). If the color tone of either the safranin or the light green solutions is too cold to be pleasing the writer adds about one-fourth per cent of auramine to the solutions, effecting a considerable improvement in the preparations.

Delafield's hematoxylin combines mordant and dyestuff, affording a very valuable histological stain. Other combinations of different mordants with hematoxylin have been proposed, but in plant histology this one answers all requirements, and is most regular in action.

Single stains are often used in plant histology and cytology when it is desired to bring out one kind of structure clearly without much reference to the associated structures. Frequently these hold a little of the stain used, of course, and are not invisible by any means. Heidenhain's hematoxylin is used in this fashion for cytological work, and safranin o for both cytological and histological work. Almost any of the primary dyes to be considered below can be used in this way if the counterstain offers no advantage. The differentiation is carried somewhat further than if a counterstain is to follow.

Combinations suited for ordinary histological work are as follows:

Safranin o followed by Delafield's Hematoxylin. Soak the sections in safranin three to twenty-four hours, or boil them in it if they will stand it. Destain with 50 per cent alcohol (acidified with acetic acid should the stain come away too slowly) until the stain ceases to come off freely and the soft tissues are simply pink against the red lignified ones. Wash in distilled water. Counterstain in diluted (about 10 per cent to 20 per cent) Delafield's hematoxylin until sufficiently dark, wash in several changes of tap water or in distilled water containing a trace of NH_4OH, then dehydrate, clear and mount in balsam. Lignified tissues, nuclei, cuticle and cork stain red; cellulose, collenchyma and chromatophores purple. Poor contrast is generally due to insufficient washing of the hematoxylin, for by washing the originally reddish stain becomes blue-purple. This stain is the most permanent of the histological combinations (unless perhaps the next) and should be depended on for research record purposes.

Heidenhain's Hematoxylin and Eosin (or Erythrosin). Mordant sections one to three hours in 1 per cent iron-ammonia-alum solution. Wash carefully in tap water for five minutes. Stain in $\frac{1}{10}$ per cent hematoxylin until sufficiently dark, or in $\frac{1}{2}$ per cent hematoxylin for one to three hours, rinse and destain to the desired degree in iron-alum solution. Wash fifteen minutes to one hour in tap water, dehydrate to 70 per cent alcohol, counterstain with alcoholic eosin or erythrosin, complete dehydration, clear and mount. Depending on the amount of differentiation, the hematoxylin is limited to the middle lamella of the xylem tissue or it is completely stained. Lignified tissues and nuclei stain black, cellulose and all softer elements pink. This combination is satisfactorily permanent, and more capable of precise differentiation when studying the structure of woody tissues alone, than the former, but more trouble and less generally useful.

Safranin o and Light Green. Soak the sections in safranin three to twenty-four hours or boil them if they will stand it. Destain with 50 per cent alcohol (acidified with acetic acid should the stain come away too slowly) until the stain ceases to come away in clouds and the parenchyma is pink rather than red. Transfer to alcoholic light green for one to ten minutes, rinse in 70 per cent alcohol, differentiate with 95 per cent alcohol, clear and mount. If the stains come away too readily, pass from light green to absolute alcohol and differentiate there. If they hold well, use more intermediate changes of alcohol so as to cause as little shrinkage as possible. Lignified tissues, cuticle and cork red, cellulose and collenchyma green. The stain is a splendid one, of brilliant contrast, and easily mastered. Not suited for tissues with little lignification. It is sufficiently permanent for ordinary class purposes, and to be preferred to the following.

Safranin o and Methyl Green. Conducted as the above. Less easy to get a clear green color, and by far less permanent in most plants. But some soft tissues appear to hold the dye remarkably well, and it seems that they are generally kinds that contain a good deal of tannin.

Safranin o and Iodine Green. The remarks just above apply equally here.

Safranin O *and Crystal (or Gentian) Violet.* Conducted as safranin-light green. Cellulose and collenchyma violet. Offers no advantages, and the violet fades more readily than light green or Delafield's hematoxylin, and offers less contrast than the former.

Safranin and Aniline Blue. This follows the general schedule for safranin and light green, but after a very brief differentiation in 95 per cent alcohol the aniline blue should be fixed and intensified by substituting a slightly acidulated (HCl) alcohol. A strong stain in safranin is necessary. After the acid treatment the sections are washed with neutral 95 per cent alcohol, dehydrated, cleared and mounted. Cellulose walls and collenchyma brilliant blue. A good contrast stain when well carried out, but somewhat more tricky than safranin-light green and not so good for photographic purposes.

Safranin O *and Orange* G. Handled like safranin and light green. A very fine stain to use for photographic purposes when strong contrast is needed between all tissues and the background without losing details within the tissues. Cellulose and collenchyma orange.

Auramine and Aniline Blue. Handled like safranin and aniline blue. More suitable for photographic work, as the contrast is not so severe nor detail in the xylem so hard to secure. It is somewhat more difficult to retain the auramine in the xylem than the safranin. Lignified tissues, cutin and cork, bright strong yellow; cellulose and collenchyma bright blue.

Iodine Green and Acid Fuchsin. Soak for several hours in iodine green, or boil in it if the sections will stand it. Destain very briefly with 95 per cent to 100 per cent alcohol. Counterstain with acid fuchsin (often best diluted with 1 to 5 parts of 70 per cent alcohol) for two to three minutes. Rinse and differentiate with absolute alcohol. The difficulty lies in losing too much of the green. Lignified tissues, cuticle and cork green; cellulose and collenchyma pink. The stain fades rather readily, but lasts two to five years if protected from light. It is invaluable in providing a stain with the colors reversed from the usual safranin-light green to use where the xylem is too dense to photograph well when stained red and to dispel the idea afflicting classes that xylem is necessarily red and parenchyma green.

Bismark Brown and Light Green. Stain lightly with bismark brown, destain with 50 per cent alcohol, counterstain with light green for one to five minutes, differentiate with 70 per cent alcohol, dehydrate, clear and mount. Lignified tissues, cuticle and cork brown; cellulose and collenchyma green. Not a stain capable of sharp differentiation, but satisfactorily permanent and excellently suited to photographic work.

Picric Acid and Methylene Blue. Stain twenty-four hours or more in saturated aqueous picric acid, rinse quickly in water and stain briefly in aqueous methylene blue. Rinse again, dehydrate rapidly, clear and mount in balsam. The lignified tissues, cuticle and cork should be lemon yellow, the cellulose and collenchyma bright blue. This is a difficult stain to handle successfully, but is useful for photographic purposes.

Crystal (or Gentian) Violet and Orange G. Handled like the safranin light green. A brilliant stain when successful, but somewhat erratic. The gentian violet will come away rather readily. Lignified tissues, cuticle and cork violet; cellulose and collenchyma orange.

In cases where differentiation with an aqueous or alcoholic counterstain cannot be accomplished because of too great loss of stain from the tissues, the material may be dehydrated to 95 per cent alcohol, differentiating the primary stain as well as possible. Then it is cleared with a solution of the counterstain dissolved in clove oil. Suitable dyes are crystal or gentian violet, erythrosin, light green and orange G. If these dyes go directly into solution too slowly, they may be moistened with absolute alcohol or about 20 per cent of this added to the clove oil. The stock solutions should hold about 1 per cent of the dye, but this may have to be diluted, especially with the gentian violet. This method of staining seems to simply "paint" a coat of the dye on the unlignified or other unstained tissues, and while it is often very brilliant it cannot be trusted too closely as to differentiation. See remarks under cytological methods (p. 159).

Combinations suited to ordinary cytological work are as follows:

Heidenhain's Hematoxylin Method. This is the finest stain available for cytological work. It is adaptable to most structures, exceeds all others in precision on chromatin-containing elements and many other parts, and in general dependability and permanence far outdistances all others. No student of plant cytology can afford to omit a thorough training in the use of this stain and in the interpretation of the results it produces. (For directions see pp. 119, 436.)

Following Heidenhain's hematoxylin one may use orange G, erythrosin or light green as counterstains, either in alcohol or in oil, but rarely to advantage. The process calls for one to twelve hours mordanting in the iron-alum, a thorough washing for five to fifteen minutes in changing water, one to twelve hours in hematoxylin, then rinsing, differentiation in the original iron-alum solution or a weaker one if better control is needed, a thorough washing for fifteen minutes to one hour in changing water, slow to gradual dehydrating and clearing and finally mounting in balsam. Differentiation should be completed under observation. If the approximate time is determined by a trial the bulk of the slides can be carried through the destaining in wells to within a very few minutes of the correct point. Then the iron-alum can be replaced by clear water and the slides removed in pairs and placed face upward in a Petri dish of iron-alum on the stage of a compound microscope where the process can be completed under direct control with the 16 mm. or 8 mm. objectives, following with the usual washing.

Safranin O-*Crystal* (*or Gentian*) *Violet.* This stain is more readily accomplished than the next, but not capable of such exact and beautiful differentiation. Stain sections in safranin for three to twenty-four hours, destain for five to thirty seconds or more in 50 per cent alcohol, counterstain in aqueous gentian violet for ten seconds to a few minutes, partly differentiate and dehydrate rapidly with 50, 70 or 95 per cent alcohols, clear and complete differentiation in clove oil. This may take some time, an hour or more. If too much stain is lost with this alcohol series reduce it to the 95 per cent alone, or even replace that by 100 per cent. Wash through 2 or 3 changes of xylol and mount in balsam. The length of time that the safranin should be allowed to destain will be found very variable and a critical feature, and will depend on the time the sections are left in crystal violet as well as the character of the material and of the safranin. It may be necessary to acidify the alcohol. Nucleoli and chromosomes stain bright red; resting nuclei (except nucleoli and heavy chromatin granules) deep violet; prophase and late anaphase nuclei with purple spireme show chromatin granules red, if present; cytoplasm very light violet; spindle darker violet. The stain does very well after chrom-acetic mixtures, where there is little advantage in the following triple stain. It is possible to substitute crystal violet in clove oil for the aqueous solution, giving a more brilliant violet but with little differentiation beyond the nucleoli and chromosomes. It should be used quite dilute (1:10) and any deposit of crystal violet removed with clear clove oil. This method and the next are rendered unsuited to very critical work on the structure of the spireme and of the chromosomes by reason of the violent changes in alcohol concentration made necessary by the rapidity with which the stains are extracted from the paraffin sections.

Safranin O-*Crystal* (*or Gentian*) *Violet-Orange* G. (*Flemming's Triple Stain*). This beautiful combination has had a great vogue. Undoubtedly very effective when correctly done, it is difficult to master. The violence of the alcohol changes necessary are quite out of the spirit of modern cytological methods, which strive to avoid any changes likely to disturb the cell structure, and the method is not suited to studies on the structure of the spireme or of the chromosomes or of any other cell organs easily disturbed by the convection currents or shrinkage. It is not possible to give any precise schedule for this any more than for the previous method. Its flexibility over a wide range of material is very great, but for any given piece of material it requires a very exact schedule: its flexibility under given conditions is very low. It is not a suitable stain to use after Bouin's solution or

mercury mixtures unless the sections have been treated with chrom-acetic fixing solution (or once-used Flemming's fluid) for twenty-four to forty-eight hours properly to mordant them. It is peculiarly adapted to chrom-osmic-acetic fixed material. Sections should be stained in safranin for six to twenty-four hours. They are then destained in 50 per cent alcohol until safranin no longer comes away freely, but remains in the nucleoli and chromosomes. If even these lose the stain the material is not suited to this method. The sections are then immersed in aqueous crystal violet for thirty seconds to ten minutes. The violet should have time to replace the safranin in all except the chromatin-containing structures. The sections are then rinsed in water and immersed in aqueous orange G to differentiate the violet. The time should be short; fifteen to sixty seconds perhaps. Then the slides must be rapidly dehydrated, differentiated and cleared in clove oil. The dehydration must frequently be accomplished with one to two changes of 95 per cent and 100 per cent alcohol, that the stain be not lost. The ultimate product should show red nucleoli, chromosomes and chromatin granules, violet spindle fibers and plastids, violet linin in the nucleus and pale buff-gray cytoplasm. Orange as such will only appear slightly, and in the cell walls. Its primary function is to sharpen the differentiation of the other two dyes. The general effect is not as gaudy as the preceding combination, but the precision of detail should be much greater. The use of oil solutions of crystal violet and orange G is to be avoided; the result is not so accurate nor is the method in the spirit of the original combination.

The methods given above represent the only ones in general use for nuclear structures and division. In addition three others may be described as having real advantages under certain circumstances.

Crystal (or Gentian) Violet and Safranin O. This method reverses the effect of the safranin O-gentian violet combination given above. It is valuable educationally in demonstrating that the position of stains is not necessarily fixed by the chemical nature of the cell organs, but may be interchanged by reversing their position in the schedule. However, often one sequence is far preferable to the other. In this instance stain in alcoholic crystal violet for six to twenty-four hours, destain very briefly with 50 per cent alcohol, counterstain with safranin (eosin can be used, or orange G, the latter primarily to differentiate the violet without itself notably appearing) a very short time (fifteen seconds to five minutes). Rapidly dehydrate, clear with clove oil, wash with xylol, mount in balsam. Chromosomes and nucleoli deep violet, cytoplasm and spindle red.

Cyanin and Erythrosin. The stains should be in 70 per cent alcohol. Stain the sections in cyanin for a few minutes, or if the stain comes away too readily extend the time to one or several hours. Rinse the sections in 70 per cent alcohol very quickly and stain for a few seconds in erythrosin. Then 95 per cent and 100 per cent alcohol. Clear in clove oil, to xylol, and mount in balsam. If the stains come away too readily omit the 95 per cent alcohol and the clove oil. Chromosomes blue; cytoplasm pink. As a histological stain, lignified structures blue, cellulose pink.

Methyl Green and Acid Fuchsin. These can be used as aqueous solutions, staining in the green for a few hours, destaining in water or 50 per cent alcohol until the dye is largely removed except from the nucleoli and chromosomes, and then counterstaining for a very brief period in the fuchsin: thirty seconds to three minutes. After staining dehydrate with 100 per cent alcohol, clear in clove oil, to xylol, and mount in balsam. The fuchsin will drive the green out of the chromosomes unless the time is short, and both stains come away very readily. Chromosomes and nucleoli green; spindle fibers and cytoplasm pink.

Latex and latex vessels in plants are preserved by methods already given (p. 111). Sections are generally cut longitudinally. Stained in a dilute aqueous iodine solution followed by aqueous eosin or erythrosin, they should show the tubes as rose-pink structures containing rich purple

rounded or femur-shaped starch grains (as *Euphorbia tirucalli* or *E. splendens*), and may be mounted in 2 per cent acetic acid or dehydrated, cleared and mounted in balsam, but the stain of the starch cannot be made permanent. If the sections (*Ficus elastica*, etc.) are treated with absolute alcohol various soluble substances are removed and the caoutchouc left. Latex vessels (*Tragapogon*) are best shown by a rather heavy stain of safranin, subsequently dehydrated, cleared and mounted in balsam. Latex cells (*Sanguinaria*) also are best treated in this way.

Sieve Tubes. Material should be preserved as indicated on p. 111. Sections cut transversely will show the faces of the plates, cut longitudinally, the relation of the contents to the pores, but in the average plant they are not easily shown. For demonstration to classes Curbitaceae give suitable material. *Cucurbita Pepo* should be stained for twelve to forty-eight hours in a 0.001 aqueous solution of aniline blue, rinsed and dehydrated to 70 per cent alcohol, counterstained with 1 per cent eosin or erythrosin in 70 per cent alcohol, the dehydration completed and the sections cleared and mounted in balsam. This progressive stain should need no differentiation, but if desired the sections may be stained in a strong solution for a few hours and differentiated in glycerin. Stains with aniline blue can be fixed and somewhat strengthened by treatment with a mild alkaline solution (NH_4OH or Na_2CO_3). The callose of the sieve plate should be blue, as also the lignified tissues, and the rest of the preparation pink.

Sperm in plants if abundant are in general most readily fixed in a drop of water on a slide, inverted over a bottle containing osmic acid either solid or in solution. The sperm are quickly and nicely fixed by the vapor, which dissolves in the water in which they are suspended. These slides can then be dried and stained with iodine green and acid fuchsin, Delafield's or Heidenhain's hematoxylins, Flemming's triple stain, etc. If the cilia show with difficulty, Loeffler's flagellar stain (p. 96) or carbol-fuchsin (p. 92) may be tried. The large sperm of Cycads are best fixed in the enlarged ends of the pollen tubes before they are shed, and sectioned (p. 178).

IV. Microchemical Reactions in Plant Membranes

Quite apart from the use of microchemical methods to determine the nature of the walls of plant cells is their use to differentiate tissues from each other for purely histological purposes. The reactions involved rarely give permanent preparations. For the more specialized procedures the section on microchemistry should be consulted.

Cellulose is probably the wall material most commonly met with among higher plants. It is frequently impregnated with other substances. A good reaction is generally secured if sections are soaked in a solution of 0.3 per cent iodine and 1.3 per cent potassium iodide in water, following this with a few drops of 60 to 70 per cent sulphuric acid. The cellulose turns blue, the lignified tissue becomes somewhat more yellow than before.

Lignin is one of the commonest tissue-modifying substances replacing cellulose. A solution of phloroglucin followed by 50 per cent hydrochloric acid gives a splended red-

purple coloration of such tissues. Aniline sulphate (saturated aqueous) followed by sulphuric acid gives a good golden yellow color to lignified tissues.

Cutin is generally deposited on the outer wall of the epidermal cells of aerial parts of plants. In the case of xerophytic plants it may become quite thick, as on the leaves of *Aloë, Gasteria,* or of *Hedera.* It stains with various substances, such as chlorophyll, alkanin and sudan III, and in ordinary histological practice with those that also stain cork and wood.

Suberin is the typical waterproofing substance in cork, and appears elsewhere in plant tissues. When present the tissues become comparatively insoluble in sulphuric and chromic acids, becoming invisible in the latter because of its dark color, but dissolving only after some days of exposure. Suberin gives the staining reactions described above for cutin. Neutral violet (1 : 10,000 aqueous, slightly acidified) is reported to stain cork violet, but not to stain cuticle. Like cutin, cork turns yellow-brown after chlor-zinc-iodide in sections which have been treated with javelle water and washed with 1 per cent hydrochloric acid, while cellulose turns violet.

Pectin appears principally as a calcium salt in the cementing substance in tissues of higher plants, joining the cells together. It readily dissolves in macerating fluids (121), or, in fruits, even in simple boiling water or very dilute acids. It stains brown-red with neutral violet (above), and also with ruthenium red, which also stains gelatinous derivatives.

Mucilage and gelatinous walls are readily stained by methylene blue and gentian violet. (See the method designed by the writer for algae p. 166.) "Slimes" may to some extent be differentiated by their color reactions, but in nature are generally mixtures. Cellulose-derivatives hardly react to iodine and stain with congo red in an alkaline solution. Pectose-derivatives are coagulable with lead-acetate and with alum, and become brown with iodine. They stain with hematoxylin, methylene blue, neutral red and ruthenium red in neutral solutions. Callose-derivatives dissolve with dilute sodium and potassium hydroxides very readily, and color with aniline blue in an acid solution.

Chitin to be recognized must first be freed from other substances. The material is sealed in a glass tube in glycerin and heated to 300°C. in an oil bath. It is then similarly treated for twenty minutes in 60 per cent potassium hydroxide at 160° to 180°C. and washed on an object slide with absolute alcohol. From this it is brought to distilled water and stained with aqueous iodine and then with 1 per cent sulphuric acid, when the chitinous walls stain red-violet. In 70 per cent acid the color disappears, but if cellulose is present a blue color develops in walls of those tissues.

Callose generally appears in the sieve plates of phloem tissue, and also in pollen grains and tubes, and the mycelium of fungi. It is very strongly colored by a dilute aqueous solution of corallin in 4 per cent soda, or by dilute aqueous aniline blue followed by dilute hydrochloric acid.

Silica can usually be recognized after incineration of the sections, the siliceous portions remaining comparatively unaltered. The silica can be removed with hydrofluoric acid (p. 113).

V. Mounting Methods

1. **The refractive index** of the mounting medium chosen is of considerable importance to the botanist because so much material is studied unstained. If it is desired to exhibit the structure of delicate colorless parts a medium with a refractive index either much higher or much lower than the specimen should be chosen. On the other hand, if the object is dense and it is desired to make it more transparent a fluid of equal density should be chosen. Of course the nature of the specimen must be considered: sometimes aqueous media must be used, and sometimes oils or resinous sub-

stances. A great deal can be done to decrease the effect of obstructive portions of a specimen by careful selection of the mounting medium. For direct recommendations consult the sections describing the characters of the different media and the special section appropriate to the plant under consideration.

The following table has been adapted from Schneider-Zimmerman.* Those media marked with an asterisk require dehydration.

Substance	Refractive Index Approx.
Air	1.000
Water, distilled	1.333
Alcohol, ethyl	1.361
Formaldehyde, 40 per cent	1.372
Glycerin, 50 per cent aqueous	1.400
Lactic acid	1.441
Glycerin gelatin	1.447
* Linseed oil	1.470
Glycerin, pure	1.475
* Paraffin oil	1.481
* Xylol, toluol	1.495
* Cedarwood oil, clearing	1.504
* Cedarwood oil, immersion	1.515
* Clove oil	1.535
* Euparal	1.535
* Wintergreen oil	1.536
* Venetian turpentine, conc	1.542
* Canada balsam, hardened	1.547
Zinc iodide, sat. glycerin sol	1.560
* Styrax, hardened	1.580
* Balsam of Tolu, hardened	1.640
* Monobromnaphthalein	1.661
* Hanna's synthetic resin	1.800

2. **Aqueous mounting media** as used for plant epidermis, hairs, fibers, pollen, spores, unicellular or filamentous algae or other small objects generally give slides of a fair degree of permanence if the cover glass is suitably sealed to the slide (p. 140).

Parts of moderate thickness had best be placed with the fluid in a ring-like cell built up of several well-dried coats of cement and the cover sealed to this. The fluid used may be a 4 per cent aqueous solution of formalin, 2 per cent aqueous acetic acid or 10 per cent aqueous glycerin (or a stronger glycerin if the object will stand it). Marine algae if mounted from the living state should be put into a solution compounded with sea water, rather than distilled water. Lime impregnated material (as Characeae) must not be sealed in acetic acid, and is safest in glycerin.

* Schneider-Zimmerman. Botanische Mikrotechnik, 1922.

It is possible to preserve tolerably well the color of many green algae, including Conjugales, Ulotrichales etc. The simplest fluid consists of concentrated formalin which is saturated with copper acetate. If this is diluted to about 4 per cent it makes a very satisfactory preserving fluid for blue green or green algae in which they may be kept in bulk or in which they may be mounted on slips. The green color will shift to a somewhat more bluish cast than the original, and some shrinkage may occur. A formula offered by E. D. Evans follows: Fix in 5 per cent formalin (neutral) 10 c.c. and 10 per cent zinc acetate in thymol water 1 c.c., diluting if shrinking appears. The material is washed after a time and preserved in glycerin by concentration. Another method by Keefe is notably free from tendency to shrink and maintains a natural green color: 50 per cent alcohol 90 c.c.; commercial formalin 5 c.c.; glycerin 2.5 c.c.; glacial acetic acid 2.5 c.c.; copper chloride 10 gm.; uranium nitrate 1.5 gm. This serves as a fixing and a preserving fluid combined. For Myxophyceae 10 gm. of copper acetate may be substituted for the copper chloride plus uranium nitrate. The presence of alcohol will prevent the use of this fluid in microscopic mounts but the material may be transferred to a mixture from which it has been omitted.

Much botanical material of a type demanding mounting in aqueous media cannot be stained, and for its most perfect exhibition should be mounted in a medium of a higher refractive index than those listed. In general the material should be brought into these more dense media by suitably graded changes. The most generally used fluid will be concentrated glycerin. The material may be placed in a 5 per cent to 10 per cent solution and this concentrated by evaporation in a warm place. A solution (Lactophenol) composed of lactic acid 20 gm., carbolic acid (crystals) 20 gm., glycerin 40 gm., water 20 gm. is very serviceable and may be used for mounting various materials, softening dried material (especially algae) or decalcifying specimens. Zinc iodide saturated in glycerin gives a solution of very high refractive index.

Stains suited for use on material to be mounted in fluid are not very numerous. Preserved material may be permanently stained in 1 per cent aqueous eosin solution, differentiated in distilled water, the stain then fixed with 2 per cent aqueous acetic acid, in a fresh portion of which the material may be mounted. The nuclei and chromatophores will be bright pink, other cell contents paler. It is possible to transfer material to very mildly acidified 10 per cent glycerin and concentrate this, but the stain usually fades. Preserved material may be carefully stained in Delafield's hematoxylin, well washed in tap water and mounted in dilute or concentrated glycerin. Cellulose membranes will be light purple, nuclei and chromatophores darker; the stain is fairly permanent. Preserved material may be mordanted in ½ per cent aqueous iron ammonia alum, washed, stained in ½ per cent aqueous hematoxylin, rinsed and exceedingly cautiously

destained in ½ per cent (or even more dilute) iron ammonia alum, washed and mounted in dilute or concentrated glycerin (see below). The stain is permanent; nucleoli and pyrenoids stain black, nuclei and chromatophores gray. It is highly satisfactory and on many forms (such as *Spirogyra nitida*) strikingly effective.

Since in fluid material it is difficult to maintain the orientation of unicellular organisms and since many fluids dry out if carelessly sealed, a series of solid media have been designed. The most commonly used is termed glycerin jelly and is applied in a melted state. When a large amount of material is to be mounted the bottle may be kept in a hot water bath, but for occasional use it is better to melt small blocks of the jelly on each slide and add the material to the melted medium, thus avoiding a continuous decrease in the gelification power of the gelatin. A satisfactory formula consists of high grade dry gelatin 15 gm., distilled water 60 c.c., glycerin 70 c.c., carbolic acid about 1 c.c. The carbolic acid and the glycerin should be mixed and added to the gelatin dissolved with heat in the water. No ordinary stains can be depended upon as permanent in this medium. Material which shrinks easily should be transferred from strong glycerin.

Cements for sealing aqueous microscopic mounts of plant tissues, fibers, etc., in watery solutions, glycerin or glycerin jelly are important factors in assuring permanence of these preparations. The cover glass and the slip about it must be scrupulously clean, the faintest trace of glycerin causing the slide to deteriorate rapidly. Thin coats of the cement are brushed over the margin of the cover and the adjacent slip free-hand or with the aid of a turn-table, each being allowed to dry before its successor is applied. Various substances and mixtures have been suggested, including vaseline, shellac, Canada balsam, marine glue, King's cement, gold size, asphaltum, etc. A particularly satisfactory medium suggested by Hazen is composed of rosin 8 parts and anhydrous lanolin 2 parts, the rosin being melted first and the lanolin then added. This may be applied hot to the edges of the cover glass with a glass or metal paddle, or dissolved in benzol or toluol and applied with a brush. It does not crack or become brittle under ordinary conditions. If it is to be used to seal slides kept at a low temperature the amount of lanolin may be raised, or if used in the tropics it may be decreased.

3. **A non-drying oil** is sometimes a desirable mounting medium. For this purpose white mineral oil or paraffin oil is most suitable. It requires dehydration and clearing of the material through xylol, benzol, etc. Mounts in this may be sealed for a time with glycerin jelly or glue. Oil of wintergreen is rather better in that clearing is not necessary, but it has a higher refractive index. The synthetic form, methyl salicylate, is quite satisfactory and may also be used for clearing injected stems, leaves, etc. for gross anatomical observations. Castor oil, linseed oil, olive oil may also be used for temporary mounts.

4. Resinous mounting media serve in general the same purposes for plant and animal tissues. First in popularity is Canada balsam. As sold for student use in collapsible tubes this is often, even generally, simply the natural resin filtered free from debris. It contains turpentine and other substances harmful to stains and what is even more troublesome, oils that evaporate slowly, so that the mounts made with this product (usually called "paper filtered Canada balsam") harden only after many months. For proper use this raw balsam should be gently heated over a flame or sand bath to a temperature that will volatilize these hydrocarbons so that the mass, when cool, is quite hard. It is then dissolved in xylol, toluol or benzol, the last evaporating rather more readily than the xylol. Chloroform is to be avoided because it fades some aniline dyes. The solution used for most plant histology, especially where the sections are rather thick, should be like thick cream. For celloidin and paraffin sections, as for most zoological work, the solution should be much thinner. It should harden firmly around the edge in twelve to twenty-four hours (less for thin mounts). The universality of adoption is one of the chief recommendations of this medium. When critically inquired into it is open to serious objections. If the heating has been insufficient volatile oils remain that are dangerous to aniline dyes. Mounts at first satisfactory gradually deteriorate centripetally as yellowing and oxidation of the medium progresses inward, for this acid condition readily fades hematoxylin preparations and some aniline dyes. A peripheral third or more of a cytological mount may thus be ruined in five to ten years, and where such slides have value as records this is very serious. For filamentous plants a special technique is most effective (p. 143).

Damar balsam, or gum damar, is a very excellent and economical mounting medium, and one which may well displace Canada balsam. The crude product as packed by dealers generally consists of vari-sized lumps and powder. Most of the trash is mixed with the finer material, so that if the lumps are picked or screened out a notable saving is accomplished in subsequent clarification. A general method of preparation, then, is to discard powder and trash, rinse off the lumps with xylol, pick out any dirty ones and dissolve the clean remainder in xylol, or if a more rapidly drying reagent is desired, in benzol and filter. Some difficulty may be found in filtering off the trash, and the product may not be perfectly clear. This is not serious in itself, but as users of this mixture sometimes report a little crystallization of the medium in old slides, it may be symptomatic of other defects. The writer offers an alternative method which he hopes has eliminated both difficulties.

The crude material, preferably only the lumps, is melted over a Bunsen flame. The fluid product is poured into an ample volume of the ultimate solvent (benzol preferred) with constant stirring, to produce a quite weak solution. When the coarser trash has settled the balance is decanted

through a loose plug of glass wool in a funnel, then filtered through a couple of thicknesses of lens paper in a ridged funnel, and finally through a soft filter paper. The main difficulty in filtering seems to be due to a slimy flocculent contamination which settles with extreme slowness. However, if dilute, the solution passes very quickly through the strainers indicated and yields a perfectly clear, pale amber fluid which, since it is too dilute for most uses, should be allowed to concentrate in a warm place. If a little more of the flocculent precipitate separates out it can be discarded by decanting. A large batch of the medium can readily be prepared in an afternoon by this method. This solution is freed by the preliminary heating of any moisture which may have been contained in the resin, and the final solution is much cleaner than by the alternative method. Damar balsam offers the important advantage of resisting marginal oxidation around the preparation so that hematoxylin slides do not fade. Aniline dyes are perfectly safe also, and as damar is much less yellow than Canada balsam, blue dyes show to better advantage in thick mounts, and for photographic purposes it offers less of a color-filter action to disturb actinic adjustments. (For algae etc. see p. 164.)

For mounts which it is desired to retain in a rather soft condition for a considerable length of time raw Canada balsam may be used, or thickened cedar oil, or Venetian turpentine (p. 143). The latter does not require clearing before mounting nor does the mixture sold under the name of Euparal, which is a very useful mounting medium entered from alcohol of 70 per cent (?) or higher. Euparal does not seem to fade dyes, but is liable to cause considerable shrinkage in any plant material whose cells are not laid open by a sectioning device (as *Fucus* sporelings, algal filaments etc.). For work with diatoms where it is desired to secure the maximum refractive index possible, styrax or tolu balsams are often used. They may be prepared as damar by the cold method, and are usually dissolved in chloroform, which allows the slide to harden quickly. Recent investigations by Hanna* suggest that he has succeeded in preparing a permanent resinous medium synthetically which has a refractive index of 1.70, approaching 1.80 when hardened. It is soluble in aniline oil, and has a somewhat yellow color, but is excellent for work on diatoms or chitinous structures.

Balsam mounts are cleaned after they have hardened, when it is customary to clean around the cover glass by scraping and wiping with xylol. A better method is to prepare a mixture of equal parts of xylol and 95 per cent alcohol, and then add water to this clear mixture drop by drop with shaking, stopping just short of milkiness. If too much water is added the alcohol and xylol will separate, which requires that the bottle be shaken frequently during use. If this separation or milkiness occurs add a few drops of absolute alcohol and shake until the mixture again just becomes clear.

* Hanna, G. D. *Science*, 1927, lxv, 575.

Keep in wide-mouthed bottles and dip slides in the solution until the excess balsam is softened enough to wipe off. If the mounts are not unusually thick this fluid will not show a tendency to cut under the cover in normal use.

5. **Balsam infiltration mounting method** is an alternative to the Venetian turpentine method (below) and much preferred to it by the writer for filamentous algae and fungi, prothallia, etc., and is a method long since adopted by various Continental workers handling this type of material. The filaments are stained in Heidenhain's hematoxylin, safranin and light green, safranin and aniline blue, magdala red and aniline blue or other suitable combinations (p. 134). From aqueous stains they are placed in 5 per cent glycerin and this concentrated by evaporation. From stains in alcohol, dehydration is usually completed through alcohol. The concentrated glycerin is removed very carefully (if necessary) by several changes of 95 per cent alcohol. It usually sticks to the mass rather closely. Two or 3 changes of absolute alcohol follow, then a series of about 6 to 10 intermediates between alcohol and xylol (as: 5:1, 4:2, etc., or 9:1, 8:2, etc., the longer series being preferred). The time in absolute alcohol and these xylol mixtures can usually be reduced to five minutes or less; they are followed by 2 or 3 changes of pure xylol. It is then placed in dilute Canada, or better, damar balsam, in strength about $\frac{1}{5}$ or less of that used for ordinary mounts. This is allowed to concentrate by evaporation at room temperature or near a radiator. The transfer to the balsam is somewhat critical; this must be very dilute. The writer found greatest liability to shrinkage at this point, but the remedy is simple. Evaporation to a mountable consistency should take about two days (rarely more) for delicate material (*Spirogyra, Vaucheria*). The filaments should be dipped up with a section lifter and placed on the slips with the solution in which they have been prepared. If the volume is insufficient it may be augmented with a little more dilute (on no account more concentrated) balsam. The mounts harden quickly and are altogether permanent. Advantages over the Venetian turpentine method lie in the avoidance of the very critical alcohol-turpentine transfer with the danger from water vapor and the bulky desiccators; in the more rapid hardening of the mounts; in the wider choice of stains available; in the more ready availability of the medium and its somewhat higher refractive index, etc. This method is extremely good for all delicate material. Vegetative *Spirogyra*, for instance, should show no shrinkage, while even late conjugating stages come through exceptionally well, although it is very difficult material.

6. **Venetian turpentine infiltration mounting method** has been so capably presented and praised by Chamberlain* that special commendation is superfluous. The writer considers the balsam infiltration method to be distinctly preferable. The process follows that method to the absolute alcohol stage. The turpentine is used as a 10 per cent solution in absolute

* Chamberlain, C. J. Methods in Plant Histology, 1924, p. 101.

alcohol. Material is placed in it from absolute alcohol in an open dish and immediately put into a desiccator over soda lime. Here it becomes concentrated to a mountable consistency. The turpentine at 10 per cent is excessively sensitive, absorbing atmospheric moisture and clouding readily. The denser solution in which the material is mounted is not so sensitive. This method eliminates the xylol-alcohol stages, but has various disadvantages (p. 143). Exceedingly successful results may be achieved by it.

B. CYTOLOGICAL METHODS

VI. Choice of Methods and Standards

Choice of methods and standards by which the results may be judged constitutes the most difficult problem in cytological technique. It is no longer possible to give a concrete schedule which by minor variations can be considered as covering the requirements of studies in plant cytology. It is not enough to consider what plant is to be the subject in planning a technique, nor what organ of it, nor the particular cells and their structural positions, but one looks to the cell organs of special interest and their physiological state, usually as it may be marked by a mitotic phase or some other condition associated with a distinctive morphological picture. Since, with the newer developments in using chromosome numbers as distinguishing features in genetical work, a vast number of published data have been based on studies using technique of the most primitive character, it becomes necessary to distinguish much more sharply than has been done in the past between processes which promise to produce results of fundamental importance in pure cytology and those which only subserve chromosome counting and similar utilitarian purposes. Studies of the latter class will assist geneticists, but they will not advance the basic knowledge of cell processes. Although the difficulties of making such studies may be considerable, and although there may be physical obstructions in the way of easy observation, yet they will always rank as requiring a lower grade of effort and method, less knowledge of general cytology and less constructive imagination than studies from which knowledge of a process rather than a mere list of figures is the ultimate result.

It is not possible, naturally, to mark a sharp line of distinction between critical and applied cytology, but it is vitally important to indicate the wide range of results required and of methods appropriate to these. Material which has been prepared by methods suited to the mass production of modern utilitarian data, counts etc., that hardly more than serve to relate the plant as represented by one cell organ to other plants, is rarely, if ever, suitable as a source of information on basic conditions and changes in the cell as an organism or an organ. This cannot be too strongly emphasized. It appears to the writer most unfortunate that persons familiar with the

methods suitable for counting purposes should have so freely fallen into the erroneous idea that because the cells appear unshrunken and the chromosomes distinct at metaphase then all other cell division stages (not to mention cytoplasmic features) must be correctly portrayed. As a matter of fact this is far from true; in hardly any of the recent studies in which chromosome enumeration was the prime desideratum are the other cell features when described (chromosome form, prophase and anaphase structure and history, all cytoplasmic organs) even approximately truthfully interpreted. It is quite impossible for a man in two or three years to count the chromosomes in scores of individuals (not to say species), to do so by methods which would correctly preserve the more critical phases, and still have time to observe intelligently these conditions, interpret them in scholarly fashion and record them in a clear style. The attitude of mind which would lead him to undertake the counting of a long range of forms would prohibit the more critical labor, even though a technique may be perfected (p. 117, new smear method) which will enable him to accumulate quickly a great mass of critical material. In no case is it safe to draw even the more elementary conclusions as to the cell history from material fixed after the older style methods, involving unopened or even opened flower buds, large unopened anthers, cells in the depths of root tips or comparable conditions in other than phanerogam plants.

Zoological technique in cytology has developed for animals a set of standards of quality far in advance of those which prevailed under the influence of Strasburger and the Bonn school. It has become evident that no near approach to a correct solution of the fundamental problems involved in the organization of the spireme and the structure of the chromosome can be reached on plant material by the customary methods. As orthodox plant technique has failed to advance new lines of attack, botanists have been diverted to the less critical and more rapidly productive investigations of embryo sac history and of chromosome number. These are of great value, and they have given the botanist a tremendous advantage over the zoologist in matters of evolutionary and genetical cytology to offset the latter's lead in technique applicable to the finer details of structure. As much of the disparity in this last respect is apparent rather than real, and due to a failure to follow up certain natural lines of advance in method, it is of advantage to emphasize the present situation and to point out in the following paragraphs what seem to be the most promising lines of advance toward more perfect technical methods. Some animal groups have been extensively studied respecting chromosome number, but outside the orthoptera and perhaps mammals, there seems less group constancy than in plants.

A primary distinction must be drawn between three types of cytological study, viz. those of cytosomic, mitotic and nuclear elements. First come those involving the general structure and the vegetative organs of the

cytoplasm. These methods involving the structures (or appearances) in plant cells called Golgi apparatus, mitochondria, chondriosomes etc., are studied by methods not essentially different from those used on animal cells, for which most of them were designed. Consequently reference to these methods should be made (page 198).

1. **Flagella** are generally most readily demonstrated by methods adapted equally to the motile stages of algae or to protozoa. Dark field illumination is highly effective. Fixation of organisms in a small hanging drop by osmic acid vapor followed by drying of the sample and staining by Loeffler's bacteriological technique (p. 96) is often effective. Simple fixing and staining under a cover glass with aqueous iodine is also often effective.

2. **Protoplasmic connections** (**Plasmodesmen**) between cells are generally made more evident by the swelling of the walls. They are most suitably demonstrated in algae (p. 164) or in endosperm of seeds, sieve tubes, cortical tissues of some gymnosperms, etc. They may be fixed adequately with simple 1 per cent osmic acid and stained with crystal violet or pyoctanin (3 per cent aqueous). If the walls are to be swollen this may be effected with 25 per cent (or stronger) sulphuric acid to which iodine may be added.

3. **Chromatophores** (including chloroplasts and leucoplasts which have essentially the same structure) are legitimately discussed here. In studies attempting to trace their origin from mitochondria-like bodies methods from (p. 198) should be selected. Comparatively little information as to chromatophore structure appears to derive from preparations using the standard Flemming's fluids, Bouin's and Gilson's mixtures etc., but they are quite satisfactory for studies on chromatophore form, especially the first group, provided the fluid acts quickly enough to prevent contraction of the plastid.

4. **Pyrenoids** show quite satisfactorily after these same fluids and their position in relation to starch grains, etc., may be determined. Material fixed in saturated alcoholic bichloride of mercury and thoroughly washed may be stained for twenty-four hours in 0.2 per cent acid fuchsin, when the pyrenoids should be bright pink, the nucleoli by contrast not stained. As the chromatophores are quite responsive to toxic agents it is essential that killing be very rapid to prevent these contraction alterations. After fixing, washing and dehydration follows a moderately close schedule (pp. 157, 113). Some pertinent data have recently been presented by Zirkle* who has worked with monochromatic light on living chromatophores.

5. **Eleioplasts** may be fixed like chromatophores, but special methods have been suggested. Material fixed in saturated aqueous picric acid is stained in a solution of aniline blue in water which has been turned from dark blue to a Delafield's hematoxylin-like purple by the cautious addition of alkanin. After several hours the eleioplasts should show purple, con-

* Zirkle, C. *Amer. J. Bot.*, 1926, xiii.

trasting with the light blue of the plasma, the dark blue of the nuclear chromatin and the red of oil droplets. In neutral glycerin gelatin the colors hold fairly well. As the exact method of functioning of these structures is obscure there is need of more intensive study of them, and the student should refer to the literature for other methods.

6. **Polar caps, spindle fibers** developing **cell plates, asters, centrosomes** and **blepharoplasts** constituting a second type, come in a group of cytological structures that appear during cell division. The last two are often peculiarly responsive to osmic acid, failing to be visible, for instance, in chrom-acetic preparations, but with the osmic acid coagulating firmly. A good example is the spermatogenous cells of *Ginkgo*, where the big spherical bodies that organize the blepharoplasts stain deep black after chrom-osmo-acetic but only as empty cavities after chrom-acetic. On the other hand polar caps and spindle fibers are of reduced visibility in preparations where osmic acid is present in large amount. The fibers are probably excellently preserved. The trouble lies in the fact that in these the cytolymph is much more completely precipitated than in the absence of osmic acid and so the fibers no longer stand out against a contrasting background. If studies are being made on the organization of the spindle by a chrom-osmo-acetic fluid then one should reduce the osmic acid as far as possible, following fixation and washing, by dehydration and clearing with a moderately close schedule (p. 113). If the reduction of osmic acid is carried to excess, however, the cytoplasm will appear largely as a mass of coarse vacuoles due to the disappearance of the most bulky portion of the cytoplasm. The irregular coagulation of the remainder will distort the fibers and give a false impression of the positions of these and of the other cell contents. Especially during mitosis and meiosis is this important, because lacking the support of the more completely coagulated cytolymph the chromosomes become clumped together. This appears to be the reason why persons working with plain chrom-acetic solution or other fluids that incompletely fix the cytoplasm (which are often used in chromosome-count studies because of the ease in getting a brilliant stain) report that the chromosomes are closely clumped together even when the cell offers ample room for the metaphase plate to spread out.

7. **Nuclear and chromosomal structures**, the subjects of the third type of study, can in certain features be preserved well even though the cytoplasm is badly fixed. On the other hand many elements of cytoplasmic structure cannot yet be preserved by any methods suitable for nuclear organs, although conserved readily enough by their own special technique. The feature of cell construction which is most notably upset by imperfect cytoplasmic coagulation is the space relation between the formed elements. For instance, unless the cytoplasm is well and finely precipitated the chromosomes tend to clump together, as was mentioned above. This is a rather obvious phenomenon the danger of which is far too often dis-

counted. The little rounded chromosomes which are usually reported as practically touching probably do not normally approach each other closer than to one-half their own diameter. Completeness of fixation of the cytoplasm avoids this artifact and enables counts to be made much more readily.

Organic adjuvants, especially sugars of which maltose appears the most adaptable, seem to assist in preventing clumping (p. 119). They also assist in maintaining the space between longitudinally cleft chromosome and spireme halves, and have contributed toward a clear recognition of the chromosome tetrad in plant cells. With the clumping of the chromosomes is associated a tendency for their contraction, most visible of course, longitudinally. While obviously more rigid than the surrounding materials, they are distinctly responsive to changes in the fixing fluids, and readily contract if slowness of penetration or incompleteness of coagulation enable them to do so. There is no proof that this effect is necessarily equal on all chromosomes in a given complex, so all studies involving a comparison of chromosome lengths must be conducted with the utmost precaution to prevent this contraction. It is not practicable to judge this directly, for the absolute lengths of chromosomes of a given kind vary from cell to cell even within a single kind of tissue depending on the stage and rapidity of mitosis or other factors, and even more between different kinds of tissues. The easiest guide to a recognition of such a change is the critical observation of the state of the fiber attachment or other constrictions, and of the satellites. If these are quite distinct, and the fibers attaching the satellites are not shortened, there is good reason to feel that the chromosomes themselves are in normal condition. Even for this some standard of excellence is necessary, but a matured experience is less likely to err than in judging contraction directly, where the normal for a given kind of cell may vary greatly.

To what degree constrictions of various kinds are a normal chromosome character is not wholly clear, but it may be assumed as probably correct that they are present at least in any chromosome large and long enough to exhibit them at the region of fiber attachment and in frequent cases elsewhere. They certainly are the morphological expression of one phase of a longitudinal differentiation of the chromosome, and experiments with chloral hydrate have demonstrated a rather extensive, if ordinarily obscure, continuance of this differentiation (p. 155).

Furthermore, direct observation of living cells may be employed (in favorable cases) to gain an idea of the natural conditions. It is worth while, however, to direct a warning remark against undiluted faith in observations on unstained (including living) material, or material under weak intravitam stain. The technique of such observations is discussed elsewhere (pp. 39, 152). The difficulties of interpretation are great, and require a special training not furnished by experience on stained material in resinous media.

The remarks that have been emphasized on the importance of a complete fixation of the cytoplasm for general studies and particularly for studies on the chromosomes during cell division apply well regarding the nucleus in maintaining the relation between its parts. The following statements, although specially adapted to such laboratory types as *Allium*, *Galtonia* or *Fritillaria* and to root tip cells, are really quite generally applicable. It is perfectly obvious on an inspection of the drawings accompanying a great many recent cytological studies that the fixation of nuclear material was limited to the nucleolus and the spirem or its representative granules. The rest of the nucleus in the fixed preparation is empty. It is far from this in the living state, being filled with a quite coagulable karyolymph. To expect the chromatic elements of the nucleus to maintain their proper relative positions without the support of this matrix would be obviously absurd. It must therefore receive careful consideration in judging the value of the fixing fluid used. If the precipitation is too dense it will be hard to secure a satisfactorily differential stain of the finer chromatic elements. Therefore the karyolymph should be coagulated in a fine-grained soft mass, rather than the heavy refractive waxy mass which comes, for instance, from an excess of osmic acid. If this is properly effected very little displacement of the contents of the nucleus will occur. It will be found that the apparent peripheral state of the granules of the resting nucleus and of the early spirem stages is only an artifact and that they are generally disposed through the nucleus.

The nucleolus will not show a bubbly structure, but will usually exhibit a smooth texture with sometimes one or more darker structures. It will not be surrounded by a clear space, which is simply due to the shrinkage of the other nuclear contents from it, generally because of inadequate fixation of the karyolymph. This artifact is in itself conclusive proof of bad preservation of the nuclear contents. Inspecting the spirem one is able to recognize its duplex character far forward into the earliest stages, in fact probably as far as its thread-character can be traced. In somewhat later stages segmentation of the spirem and the first traces of the constrictions can be seen. In the cytoplasm the polar caps will be recognizable and the clumping of the chromosomes usually associated with the breaking down of the nuclear membrane will not be very great.

The amount of differentiation which it will be possible to demonstrate within the metaphase or anaphase chromosomes is problematical and more within the province of a research paper than a general introductory statement such as this. With discretion respecting the fixing fluid and the infiltration, it should be possible (especially with a curtailed hematoxylin staining schedule) to recognize a good deal of specialization. It appears probable that at least in the larger chromosomes in vegetative anaphase there are a pair of straight or spiralled threads sometimes bearing chromomere granules, but a full interpretation of these structures is wanting.

A few further general suggestions may be offered respecting cytological criteria in meiotic and post-meiotic material. The hematoxylin smear method (p. 116) has confirmed several features of chromatin distribution previously in more serious question, so that the general standard to be demanded of preparations of such stages is higher than a few years ago. In the resting stage and very early prophases the chromatin material usually does show a general distribution throughout the nucleus, even in the absence of a good preservation of the karyolymph. Since that substance is more readily coagulable at this time than later, many kinds of cells show quite respectable preservation in a fluid that is not adequate for later stages. However, very careful attention to this phase and the use of a fluid more effective with the karyolymph notably reduces the confusion and indefiniteness of distribution of the very early spirem and apparently granular stages and offers good prospect of an eventual complete interpretation of them. It is to be expected that a spirem will be recognizable far into the prophases toward the period of the first concentration of the chromatin. This spirem (at least in large spore or pollen mother-cells) will be seen to be double. It is to be hoped that in later stages (strepsinema etc.) more cases of a tetrad structure will be demonstrated than are at present known. It is possible to expect a great reduction or even (in cells not too resistant to fixing fluids) an elimination of the contraction feature known as synizesis (by botanists often "synapsis") which represents simply the collapse of the spirem by reason of lack of support of the coagulated karyolymph at a time when the spirem is particularly slender and delicate. The conditions proper for a truthful exhibition of spirem and pre-spirem conditions (if the latter ever exist) are notably absent from most preparations of meiotic cells, for the correct preservation of them is far from the casual matter generally assumed. It must be understood that pollen or spore mother-cells discussed in the preceding paragraphs have been preserved without shrinkage of the cytoplasm, or practically so. It is almost impossible to conceive of accurate nuclear studies being made on material where even the cytoplasm surrounding the nucleus, easier by far to conserve properly, has not been able to resist malformation by a badly designed fixing fluid. If the pollen mother-cells cannot be exposed to the fixing fluid directly they will generally shrink, and in the case of small anthers or sporangia little can be done about it. Such material should simply be discarded for detailed studies.

In the case of embryosacs and deeply immersed megaspore mother-cells of conifers a comparable situation holds. It is practically impossible to get a fixation of these suitable for really critical work. What suggestions can be made are offered on page 177. The stages secured will have to be presented for what they are worth, without offering them as evidence on matters of critical importance respecting spirem constitution and behavior. It has frequently been considered by plant cytologists that they have observed

transverse division ("fragmentation") of rather elongated chromosomes, and various interpretations have been made of this process. Where there is opportunity to judge it seems that the lines of division are sharp, the ends square-cut, and not constricted across as one would expect. It may confidently be assumed that these probably always represent fractures due to imperfection of the sectioning process: too brittle paraffin, dull or ragged-edged knife, etc. It is hardly likely that they are anything but artifacts. The positions in which they appear are fortuitous rather than definite like the chromosome constrictions. Chromatin extrusion from the resting nucleus or the early spirem stages is probably also an artifact due perhaps to pressure on the tissue in cutting or some other early stage of preparation.

VII. Examination of Living Cells

The study of living cells, usually pollen mother-cells, in search of cytological data has resulted in contradictory reports, particularly with respect to the details of mitosis. Somewhat more satisfaction has resulted from the use of intra-vitam dyes, but here the actual health of the cell has been much in doubt at times. (For a discussion of the methods applicable to plant problems see p. 152.) The morphology of the colored elements of the cell, chromatophores, eyespots and the like, may best be determined in the living state, and require no special methods. Chromatophores with dilute pigmentation may be made much more evident and studied much more readily if a suitable ray-screen is placed below the condenser. A ready selection may be made from the complete and accurate series prepared by Wratten & Wainwright and distributed in the United States through the Eastman Kodak Company. It appears that an important new approach to the effective observation of living cells may result from the studies of Kuwada and Sakamura* and Sakamura.† These investigators found that the acidity of the medium in which the observations are made has a very striking effect on the distinctness of the chromosomes and of their structural elements. They found that there is a general tendency for the chromosomes to swell as the pH of the medium passed from 1.7 toward 6.7, and that this is, within wide limits, a reversible reaction. As the chromosomes contract they and their structures become more visible (in the living state) and as they swell these disappear, so that the chromosomes at metaphase appear to be represented by hollow cavities in the cell showing little content, even with dark-field illumination. Observations may be made in the viscous material from the anther cavity if that is abundant enough, or the anther squeezed into a drop of olive oil and this pressed out under a cover. In this latter method few of the pollen mother-cells actually come into contact with the oil, the rest remaining surrounded by the material from the anther cavity, even when it is small in amount. If CO_2 is passed over material under

* Kuwada and Sakamura. *Protoplasma*, 1926, i, 239.

† *Ibid.*, 1926, i, 537.

observation it is found that as the gas is absorbed by the cells the obliquely transverse banding of the chromosomes becomes increasingly evident, and as the gas is driven out of the drop by a current of CO_2-free air the chromosomes swell and the structure becomes indistinguishable. For *Tradescantia* it was found that a pH of 5.0 (or a little more acid) was about correct for a well buffered medium comparatively insensitive to the action of alkali. The anther content is distinctly alkaline and readily affects a lightly buffered suspension medium. The experimenters got the best results in demonstrating the spiral structure of the chromosomes of *Tradescantia* when the dye, neutral violet extra, was dissolved to .025 per cent in a solution of pH 4.38, and observations made on the peripheral units of the masses of pollen mother-cells. They consider that the spiral structure of the chromosomes is a natural feature easily lost from view by the swelling of the chromosomes or accentuated by their slight contraction. The spirals may be fixed in various ways, even by simple boiling water.

Intravitam stains have met with limited use on plant material due to the firm nature of the cell wall, the frequent presence of pigments, cutin and so forth. However, when the nature of the organism permits, a procedure very similar to that available for animal cells can be used. Root hairs, larger aquatic plants and aquatic algae and fungi can simply be immersed in aqueous solutions of the dye chosen. Hairs of pistils, stamens, leaves etc., and stripped epidermal layers can usually be kept in good condition and stained in an isotonic solution. Sometimes suitable staining of vascular plants can be secured by immersing the cut ends in a fairly strong solution. Perhaps this is hardly strictly "intra-vitam" staining in the cytological sense, although cells bordering the vessels do take up some dyes. If eosin is used and the plant is sufficiently tender (e. g. *Impatiens Sultani*) the entire stained shoot and leaves may be plunged in 2 per cent acetic acid in absolute alcohol to dehydrate and fix the stain and then after a few hours transferred to oil of wintergreen (synthetic) to clear the preparation. For the accurate staining of living cells only a limited choice of dyes is available, of which the most important are methylene blue or neutral red (1:10,000 to 1:100,000) and bismark brown (1:3,000). They should stain nuclei, chromatophores etc., and it will often be found that the stain becomes more intense as the cell becomes injured by the treatment (p. 395).

It is often desired to apply to plant material a stain which will act without the previous interposition of fixing and mordanting processes. The dye used is usually a toxic one and the cells do not survive the treatment. Material such as plant epidermi, *Spirogyra*, etc., may well be stained in about five minutes by a 1 per cent aqueous solution of eosin which is differentiated with water and fixed with 2 per cent acetic acid (p. 139). If desired the material may first be treated with aqueous iodine-potassium iodide solution to stain the starch, and after rinsing, the eosin may be applied. Another excellent stain is picro-nigrosin, the latter dye being dis-

solved to the desired degree (as, for instance, 1 per cent) in saturated aqueous picric acid. The material can be washed, dehydrated and mounted in balsam. The acetic acid-methyl green mixture used on protozoa also serves well for many algae (p. 396).

VIII. Preparation of Cytological Material for Fixation

This requires close attention to all possible ways of facilitating the action of the fixing fluid. Under general paragraphs appropriate to each major group of plants there have been assembled suggestions as to technological methods specially suited to them. Certain general precautions should always be borne in mind and will be discussed here. Although problems in connection with vascular plants have been used as a basis for wording these directions, the data may be adapted to other groups with little modification.

1. **Root and stem tips** should be freed from sand and loam, and if they are covered with a mucilaginous investment it should be washed away if possible. The tips should not be more than 1 to 2 mm. in diameter at the very most, and if thicker, slices should be cut off opposite sides with a very keen piece of razor blade to reduce the axial portion to about 2 mm. so that the time for complete penetration should not be unduly prolonged. The mucous layer of air roots, or in other kinds the suberized root caps, can be eliminated in this way. The stem tip should be trimmed free from the leaf bases, ramenta or hairs. The desired portion should then be cut from the plant, detaching as small a part as practicable; certainly not more than 3 to 5 mm. long. In root tips it will generally be found that there is a meristematic region just back of the apex that is visibly more milky or yellow-opaque than the cap beyond it or the region of elongation above it, and this region contains all the material that is worth while for ordinary cytological purposes. The tips should each be plunged below the level of the fixing fluid instantly after cutting; indeed if any trimming is necessary it is well to do it under the level of the fixing fluid.

2. **The study of the meiotic phases** by fixing buds, anthers or ovules intact has long been the favorite method. With the increasing requirements of modern cytology for freedom from shrinkage, from synizesis, from chromosome clumping and contraction and for preservation of the internal structure of the chromosomes, such crude methods must rapidly fall into disrepute. In very tiny flowers it may be impossible to open the buds and these will then have to be fixed entire. Hairy buds may be dipped into 50 per cent alcohol for an instant to remove the air, and this sometimes helps when there are cuticularized surfaces which cannot be cut away or otherwise removed, but after alcohol they must be quickly plunged into the fixing fluid. This treatment should be omitted before using Farmer's or Carnoy's fluids, or other formulas with a considerable alcohol content. Buds and capsules of lower plants containing air chambers should generally be placed, immersed in the fixing fluid, under a vacuum pump to remove

all possible air. If it is possible to do so the flowers should be bisected, or a piece cut off one side. A mere longitudinal incision or a needle puncture is hardly effective. If the anthers are 1 mm. or more long they should be carefully excised or the perianth trimmed from about them. At 2 mm. or more they can be cut across transversely, and if they are several millimeters long they can be cut up into small fragments, or handled by the smear methods (p. 116). For holding the fixing fluid while cutting, the writer uses a Syracuse watch glass with a layer of paraffin on the bottom. About 5 c.c. of fixing fluid is poured on the wax and into it are dropped the anthers, each cluster being immediately finely comminuted with a small, very sharp, scalpel of the size used for eye operations. The accumulated anther fragments and all extruded materials are then handled through the paraffin process by a sedimentation method like unicellular organisms, which is really very easily done. Truly superb fixation of meiotic cells may be obtained in this way, the quality approximating that afforded by the smear method (p. 116) with the advantages of ease in staining and of serial sections. The secret of success lies in bringing the cells to be studied into immediate contact with the fixing fluid. As the cells are squeezed from the anther they adhere to the cut surfaces, the walls in the sacs, etc., or even to each other so as to form considerable masses. In fact, in some plants the sac contents squeeze out in a rod-like mass when pressed from the anther, and if it is considered desirable they can be fixed, dehydrated, imbedded and cut in this form, yielding admirable results, as has been demonstrated to the writer by E. E. Carothers for *Lilium*. The exposed grains yield infinitely more accurate pictures of true conditions than do cells buried even one or two layers from the surface. In the larger fragments it is easy to trace comparatively the difference in fixation quality from these exposed cells down the anther sac to the deeper-placed cells that show (in quite the most favorable way) the orthodox fixation to which plant cytologists have unfortunately become habituated. It follows from these remarks that studies on meristematic tissues, such as root tips, should be confined to cells very near the surfaces of the pieces, not more than 2 or 3 cell layers removed from direct contact from the fixing fluid, if observations of great nicety are to be made.

Ovaries offer a worse situation. The problem is the same: to give direct contact between the fixing fluid and the embryo sac. In such ovaries as those of Liliaceae the walls should be dissected away. Then, with a very sharp razor, slices should be removed from the rows of ovules so as to lay bare the deeper layers of the nucellus. If the embryo sac is opened the contents may be lost, or, on the other hand, exceptionally favorable results may be obtained. Large ovaries may sometimes be cut transversely into thin slices, thereby exposing the ovules more perfectly than if the ends alone were cut off. In ovaries with axial placentation the axial tissue may often be split up into as many strands as there are rows of ovules and these rows then

removed and fixed individually so that correct orientation of each for longitudinal sections is obtained, and a great economy of material effected. Solid fixation of the nuclei is not necessary for studies on the development of the embryosac, and even a little shrinkage (it is almost inevitable) may be tolerated. But if a study is to involve nuclear cytology, meiosis, gamete fusions etc., then the same criteria must be applied as in studies on microsporogenesis or mitosis (p. 144).

Sporangia of bryophytes and large microsporangia of pteridophytes or gymnosperms should be cut open under the surface of the fixing fluid to facilitate immediate contact of meiotic cells and the fluid. Material not amenable to the orthodox methods should be abandoned for critical studies unless new methods can be worked out which will enable it to yield slides equal to the highest standards.

3. **Chloral Hydrate.** Various experiments by Němec, Sakamura and others have indicated that this substance exerts a remarkable influence on the mitotic phenomena in plants. If it is applied to growing roots (by immersion for various periods of time) and the tips fixed after time has been allowed (one to two days) for mitoses to occur, it will often be found (*Zea* and other grasses, legumes) that the spindle fiber and other constrictions are much more marked than normal and that polyploid mitoses, giant cells etc., have been developed. Benzine and chloroform vapors, CO_2 and various other substances have somewhat similar effects.

IX. Choice of the Fixing Fluid

It is necessary to select the class of fluid to be used on a given subject by a process of elimination, having in mind the character of the material and the type of result required. The material and its possibilities will determine (a) that the most difficult studies on nuclear and cytoplasmic structures are possible by reason of its easy adaptability or (b) that greater or less difficulties interfere and only more obvious features of structure can be studied. For this second class it is wasteful to apply delicate methods, and more or less deliberate scientific falsehoods are perpetrated when an attempt is made to interpret the results beyond the natural limitations imposed by the material. For the first class the most delicate methods and the most critical interpretations may be attempted, or if only the more obvious features are of interest then quicker, less fussy methods may be used and interpreted accordingly.

Under appropriate headings special suggestions are given for each great group of plants. Only the broader features are outlined here, in large part the angiosperms being used as a basis for the presentation. First, one must notice the amount of interference offered to the penetration of the fixing fluid. If after the preparation of the material has been completed (p. 153) thick pectic layers, cutinized, suberized or lignified membranes interpose themselves between the fixing fluid and the cells to be studied, then only

the most uncritical results can be expected, and such formulas as those of Carnoy, Farmer or Gilson (pp. 157, 420, 422) should be tried, for they are best fitted to overcome the resistance of the enveloping material. If the interference is less, and is limited to thin mucous layers, but a few layers of cells with only moderately heavy cellulose walls, a slight hairiness, etc., then (in addition to the fluids just named and with which more uncritical results are to be expected) more exacting studies may also be attempted and Navashin's, Němec's, Flemming's (strong) formulas, or Allen's modification of Bouin's fluid may be used. If the cells to be studied are, as a result of the preliminary manipulations, directly exposed to the fixing fluid, the exactness of action of the fluid is greatly increased. The results in these cases are far more under the control of the experimenter than in the others. It becomes possible to fix for a particular feature of the cell structure, a particular phase of mitosis. The student may take the more critical fluids and alter the proportions of the constituents to suit the demands of the occasion, especially with the two most adaptable, chrom-osmo-acetic and picro-formol-acetic with chromic acid. In doing this due regard must be given to the specific functions of each of the constituents as described in the general section (p. 416). It is remarkable that when the cells to be fixed are exposed directly almost all fluids function more perfectly than otherwise. Even quite violent fluids (as Gilson's) show unexpected values for demonstrating particular features of the cell. However, the writer would recommend that the investigator make every effort to apply one of the chrom-osmo-acetic group of formulas and to adjust it rigorously to the material and the problem, for he feels that these combinations are the most logical in organization and the most adaptable, and that the work of very many investigators (including those most skilled in both plant and animal cytology) has demonstrated that practically all cell structures (apart from mitochondria and a few other similar specialized cytoplasmic elements) can be better demonstrated by a well considered application of a chrom-osmo-acetic fluid than by any other so far devised. Having established by this method a certain hypothetical standard it is then advisable to try adaptations of other formulas, which often yield highly desirable short cuts to the demonstration of particular features. Delicate structures of higher plants (as developing trichomes, stripped epidermi from bulbs, etc.,) may be handled like delicate sporogenous tissues. It is notable that if the amount of vacuolization of the living protoplast is low and the fluid can act directly, formulas with very violent action (Farmer, Carnoy, Gilson, pp. 157, 420, 422) can be used without the usual great shrinkage; consequently these have had a deserved popularity in work on filamentous fungi and on cells of similar structure, such as are occasionally found among algae or developmental stages of higher plants. They should always be avoided if possible when there are one or more prominent vacuoles in each cell.

Many of the fixing fluids used in plant technique are the same as those used in animal work (p. 416). Because of the resistance offered by the cellulose or indurated walls in plant tissues it has become customary to use formulas more notable for their penetrating power and the ability to preserve unshrunken the grosser cell elements than for the precision with which they maintain the finer nuclear or cytoplasmic details. It is urgently necessary to realize that a solution giving a slide of a good general aspect may preserve in a totally untruthful fashion one or many details of cell structure, so that in cytological work the fluids should be selected and modified for very specific uses, and general-purpose formulas should be distrusted for definitive studies. The following formulas are in some cases somewhat modified from their original form. None are necessarily in the best adjustment for any given piece of work, and none should be discarded for a specific purpose without a carefully planned series of experiments involving such changes in the proportions of the constituents as their individual characteristics suggest, and also changes in the manner of immersion and the temperature of fixation (p. 184).

Bouin's Solution (p. 424). In its original form it is not suited to plant protoplasm.

Bouin's Solution, Allen's Modification (p. 183). This gives a fixation notable for the brilliancy of the staining with Heidenhain's hematoxylin, and is specially suited for the making of chromosome counts. It is not (in the present writer's opinion) suited for studies on prophase chromatin conditions. It is not suitable for use before Flemming's triple stain. It should act for ten minutes to a few hours, depending on the material.

Carnoy's Fluid (p. 422). Since it fixes by dehydration it is a fundamentally unsound combination for critical cytology; it usually produces extreme shrinkage, but may be adopted if it is necessary to penetrate hairy or cutinized surfaces.

Chrom-acetic Solutions. These solutions have good power of penetration, preserve the chromatin in its coarser aspects tolerably well, but fail to precipitate the karyolymph and some elements of the cytoplasm. Prophases are not well preserved, metaphases frequently are clumped and the chromosomes contract longitudinally. While the fluids may be used for histological purposes, for general cell topography and for chromosome counting on easy material, they are not as good as the mixtures containing also osmic acid. *Weak chrom-acetic.* 10 per cent chromic acid, 2.5 c.c., 10 per cent acetic acid, 5 c.c., water to 100 c.c. *Medium chrom-acetic.* 10 per cent chromic acid, 7.0 c.c., 10 per cent acetic acid, 10 c.c., water to 100 c.c. *Strong chrom-acetic.* 10 per cent chromic acid, 1.0 c.c., 10 per cent acetic acid, 10 c.c., water to 100 c.c. The fluids should act for a few minutes on delicate specimens, from twelve to twenty-four hours on tough ones. Washing may be done for approximately equal periods with running or frequently changed water, or during dehydration with alcohols provided the material be kept in the dark. The weak solution is suited for filamentous algae, fungi, prothallia and objects a few cells thick, the medium solution for root tips and ovaries, the strong solution for woody material and tough leaves.

Farmer's Fluids. Absolute alcohol 6 pts., glacial acetic acid 1 pt., or a stronger formula, absolute alcohol 2 pts., glacial acetic acid 1 pt. The remarks and directions under Carnoy's fluid apply here as well.

Chrom-osmo-acetic Fluids. This group of formulas represents a few of the possible combinations of chromic, osmic and acetic acids. Mixtures involving these substances give by far the most truthful representation of most of the elements of the plant cell, and a formula of the Flemming type is to be adopted if at all possible in studies on chromatin

behavior or the cytoplasmic mechanisms with the exception of mitochondria and a few other organs. It is best to keep the osmic acid for these formulas in a chromic-acid solution, for there it is more stable than in a simple aqueous solution. As, however, the addition of acetic acid or other organic adjuvants decreases the stability, it is best to make the final solutions up just before use. The formulas as given here are organized on the basis of a mixture of chromic and osmic acids. *Benda's Modification.* Chromic acid, 10 per cent aqueous, 3.1 c.c., osmic acid, 2 per cent in 2 per cent aqueous chromic, 12 c.c., glacial acetic acid, 6 drops, water 41.9 c.c. *Bonn Formula.* Chromic acid, 10 per cent aqueous, 0.33 c.c., acetic acid, 10 per cent aqueous, 3.0 c.c., osmic acid, 2 per cent in 2 per cent aqueous chromic, 0.62 c.c., water 6.27 c.c. *Weak Flemming.* Chromic acid, 10 per cent aqueous, 1.5 c.c., acetic acid, 10 per cent aqueous, 1.0 c.c., osmic acid, 2 per cent in 2 per cent aqueous chromic, 5.0 c.c., water 96.5 c.c. *Strong Flemming.* Chromic acid, 10 per cent aqueous, 3.1 c.c., osmic acid, 2 per cent in 2 per cent aqueous chromic, 12 c.c., 10 per cent acetic acid, 30 c.c., water 11.9 c.c. *Flemming for Gasteria and Smear Method* (W.R.T.). Chromic acid, 10 per cent aqueous, 0.20 c.c., acetic acid, 10 per cent aqueous, 2.0 c.c., osmic acid, 2 per cent in 2 per cent aqueous chromic, 1.50 c.c., water, 8.30 c.c., maltose 0.15 gm. This last formula has proved exceedingly valuable in studies on chromosome form and structure. It should be made up in small quantities as required, with the aid of a 1 c.c. pipette graduated to 100ths. The amount of maltose is subject to variation at the discretion of the experimenter, and seems to aid in keeping the chromosomes from clumping, in preventing satellites from fusing with the main chromosome body or segmenting constrictions from being obliterated. In general the weaker solutions are used on the more delicate filamentous plants, the stronger solutions on the more resistant ones. They should be allowed to act from a few minutes to twelve to twenty-four hours, and washed out as for the chrom-acetic series. All ordinary chromatin or cytoplasmic stains do well after these mixtures.

Gilson's Fluid (p. 420). Fixation is rapid, a few minutes usually sufficing if the objects are small.

Helly's Fluid (p. 421). It does very nicely for chromosome studies if the material is imbedded in celloidin rather than paraffin. Treat as after Zenker's fluid.

Hermann's Fluid (p. 423). This, as the other platinic mixtures, functions much as the chromium mixtures without any very marked advantages.

Juel's Fluid (p. 424).

Merkel's Fluid (p. 424).

Němec's Fluid. Chromic acid, 1 per cent, 25 c.c.; formalin, 2 c.c. Use a freshly prepared solution and fix for about six hours. Then pour off and replace with a fresh portion, to act for a further eighteen hours. The time is to be reduced for delicate organisms. This formula seems to give a very fair preservation of the shapes of chromosomes when successful, but is, in the writer's experience, somewhat erratic.

Navashin's Fluid (after Karpechenko). Chromic acid 10 per cent aqueous, 1.5 c.c., acetic acid, 10 per cent aqueous, 10 c.c., formalin 0.83 c.c., distilled water 23.67 c.c. See remarks under Němec's Fluid.

vom Rath's Fluid. Saturated aqueous picric acid, 100 c.c., osmic acid 2 per cent aqueous, 6 c.c., glacial acetic acid, 1 c.c. *vom Rath's Fluid with Platinum:* saturated aqueous picric acid, 100 c.c., platinic chloride, 10 per cent aqueous, 5 c.c., acetic acid 1 c.c., osmic acid, 2 per cent aqueous, 6 or 12 c.c. vom Rath's fluid may also be prepared with corrosive sublimate. The mixtures offer little of interest in advantage over the Flemming mixtures.

Schaudinn's Fluid (p. 399). This powerful mixture serves to fix on the slips spermatozoids, algal zoospores and flagellate types. It should be washed out with alcohol.

Stromsten's Fluid. Stock solution: chromic acid, 10 per cent aqueous, 16 c.c., acetic acid, 10 per cent aqueous, 100 c.c., water, 54 c.c. At the moment of fixing add 2 parts of this to 1 part of formalin and immediately immerse the material. No washing is required,

and material may be preserved in the residual fluid, which changes color soon after mixing. Before imbedding it may, of course, be washed out in dehydration.

Zenker's Fluid (p. 421). This formula is unsatisfactory for plant tissues in this form but may be used as Helly's Fluid.

X. Imbedding and Staining

The cytological material after fixation is generally followed by dehydration (p. 114, longer schedule), clearing through cedar oil, or better, xylol-alcohol changes (p. 114), and infiltration with paraffin (p. 126). Or, after dehydration the celloidin method may be followed (p. 126) using a slow and careful schedule without heat. Cutting is accomplished by the usual means, but especial care is needed to keep the knives in perfect condition so as not to break or dislodge the chromosomes or other cell structures unnecessarily. The thickness of the sections necessarily varies with the problem, but usually ranges from 8 μ to 12 μ for microsporogenesis studies, and 12 μ to 20 μ for embryosacs. The sections are affixed to the slips in serial order by the usual means (p. 129). Staining methods available are varied, Heidenhain's hematoxylin being the most dependable, but with a wide choice of aniline combinations (p. 132). The sections are usually mounted in damar or Canada balsam (p. 141). Minute objects not to be sectioned are handled in tubes by sedimentation, the reagents being applied in succession. Filamentous algae and fungi are also frequently studied cytologically without sectioning (pp. 164, 173).

XI. Cytoplasmic Inclusions

The methods of preserving tissues so as to retain various of the solid inclusions common in plant cells have been described (p. 112). Further treatment leading to their recognition may be briefly indicated here, but as the topics are more microchemical than cytological in their nature, reference should be made to strictly microchemical discussions for more complete information.

Starch grains freed from tissues (such as commercial starches) may be mixed with crystal or gentian violet and stained, the dye then poured off and the starch washed with saturated aqueous picric acid. The lamellae should be well demonstrated by this method. After picric acid the starch may be dried and mounted in balsam. The method may be adapted to sections, where safranin will also stain starch, as will thionin. For the simple recognition of starch aqueous iodine-potassium iodide is the customary reagent, and should not be used too strong, as the dark color will obscure the lamellar structure of the grains.

Protein stored in granular form as food reserve in cells is usually preserved by the ordinary fixing methods. Lyons blue among other stains strongly affects such material. The more complex aleuron grains are readily fixed by alcoholic mercuric chloride or picric acid. The ground substance is satisfactorily stained with alcoholic eosin, when the globoid and the crystalloid stand out by contrast.

Crystals (rhaphides, tabular and conglomerate or druse crystals) are generally calcium oxalate, more rarely of calcium sulphate or other substances. Barium chloride

solution deposits barium sulphate about the calcium sulphate crystals but not about those of calcium oxalate (p. 110).

Inulin is in solution in the cells, but permanent preparations showing the sphaerocrystals in which it is deposited by alcohol are readily made (p. 111).

Glycogen is likewise in solution. It gives a brown color with iodine (iodine 0.1 gm., potassium iodide 0.3 gm., water 45 c.c.) which temporarily disappears on warming the preparation. The substance can be precipitated in the cell. Material is preserved (e. g. fungus hyphae) for twelve hours in alcohol and then for twelve hours in 10 per cent tannic acid. It must then be very quickly washed (with the aid of a centrifuge) and stained in iron chloride solution, which colors the glycogen black.

Paramylum is a type of stored carbohydrate frequently found in lower plants. The grains are apparently homogeneous or lamellate, rounded, rod-shaped, box-shaped or ring-shaped. They do not give the starch reaction with iodine, remain unaltered in 5 per cent potassium hydroxide, but swell readily and dissolve in a 6 per cent solution.

Leucosin is a stored reserve also found in lower plants. It appears to dissolve in water when the cells are treated with fixing agents, acids and alkalies.

Fucosan forms vacuolar bodies in the cytoplasm of Phaeophyceae, which are insoluble in water and do not stain with iodine. It reduces osmic acid and the older vacuoles blacken especially quickly. The vacuoles stain readily with methylene blue. The fucosan vacuoles are fixed by 25 per cent hydrochloric acid or sulphuric acid.

Volutin forms rounded bodies in bacteria, fungi, etc. These are readily fixed by alcohol, formaldehyde or picric acid. They stain readily with fuchsin, and methyl violet, but resist eosin, and do not stain by Gram's method. They darken readily with iron-alum hematoxylin, but decolorize very readily on differentiation. Material strongly stained with carbol-fuchsin is treated with aqueous iodine-potassium iodide, when only the volutin retains the stain.

Tannin vacuoles are frequently found, especially in algae. They may be readily stained in the living state with methylene blue (1:500,000). The stain may be fixed with saturated aqueous picric acid in a few hours, rinsed, dehydrated, cleared through xylol, and mounted in balsam.

Cystoliths of calcium carbonate are occasionally found, and require especial care in preservation (p. 110). They readily dissolve in dilute acids.

Sulphur is frequently found as globules in a pure state on or in filaments of algae, bacteria or fungi growing in sulphurous waters. These globules are soluble in carbon bisulphide if the filaments are first killed, as by drying.

Resin masses are usually colored green in material preserved in solutions containing copper acetate (p. 111).

Caoutchouc and latex are treated under latex vessels (pp. 111, 161).

XII. Fats*

Sections are made either with the freezing microtome or free-hand. Free-hand sections are better for all soft plant tissues, since freezing destroys delicate cells and may change the state of cell contents. Leaves, even small soft leaves, can be folded at the midrib, rolled gently between thumb and forefinger into a compact roll and sectioned free-hand. Small seeds and spores may be imbedded in paraffin. Put the seeds into a small porcelain dish or tiny paper box, pour hot (not boiling) paraffin over them, and arrange in any desired position with a hot needle. When cooled, cut the

*Section on "Fats" by Sophia Eckerson.

paraffin cake into pieces convenient for holding and section free-hand. Hard coated seeds are best sectioned with the freezing microtome.

Neutral fats in storage organs in plants are either in the form of semi-solid globules (solid in a few plants) in a matrix of protein or are in fluid state. Those having a high proportion of oleic acid are fluid. Fats in meristematic regions are present as small droplets which may be extremely minute and difficult to identify.

Two or more types of reactions are always made for the identification of substances in sections of tissue. The three most important reactions for fats in plants are: 1, solubility; 2, staining (with sudan III or scarlet red); 3, saponification, and 4, myelin formation.

1. **Solubility.** Fats and fatty oils are insoluble in water, alkalies, and salts. They are soluble in ether, chloroform, acetone, absolute alcohol (boiling), xylol, balsam, and some other substances.

Put sections on a slip in a drop of water. Examine the cells carefully for drops having high refraction. Test their solubility in the fat solvents. Draw off the water with small strips of filter paper, adding 70 per cent alcohol at the other side of the cover glass. Then add a few drops of ether. Small droplets flow together and dissolve. Larger globules break up into small drops which flow together before going into solution. Test the solubility of the supposed fat drops in chloroform and in acetone. Lecithin, cholesterol, and the phytosterols are soluble in most of the fat solvents but are insoluble in cold acetone.

Ethereal oils and resins (also caoutchouc in latex plants) may be present in the tissues as glistening drops; or resins may be in solid masses, usually colored yellow to brown. These are soluble in the fat solvents but can be differentiated with a fair degree of accuracy by their greater solubility in certain substances. Ethereal oils are easily soluble in glacial acetic; fats and fatty oils are soluble with difficulty.

Add a drop of glacial acetic at edge of the cover glass. Ethereal oils dissolve; fats remain (ricinus oil is soluble). To other sections add a drop of chloral hydrate (5:2 in water). Caoutchouc drops swell greatly but do not dissolve (Molisch*); ethereal oils dissolve; fats remain. Resins are only slightly soluble.

Ethereal oils can be removed from the sections by microdistillation (Tunmann†) in a few minutes. Sections are placed on fine gauze or cheesecloth held between two rings and placed over a small steam bath. The sections can then be re-examined and the remaining drops tested with concentrated H_2SO_4. Resin drops or masses freed from ethereal oils dissolve partially or completely. If small granules are formed they do not flow together. Fat drops break up into smaller drops which flow together.

*Molisch, H., Mikrochemie der Pflanzen, Ed,. 2, Jena, 1921.

†Tunmann, O., Pflanzenmikrochemie.. Berl., 1913.

2. Staining.

Sudan III. This dye is insoluble in water; soluble in alcohol, but more soluble in fats and oils. This fact is the basis of its use as a fat stain. When a solution in alcohol comes in contact with fats, the dye leaves the alcohol and dissolves in the fats, coloring them yellowish red. In order to act as solvents for the dye, fats must be in fluid or semi-solid state. In the case of solid fats, a slightly higher temperature, rendering them somewhat pasty, is usually effective.

A concentrated solution (approximately 0.5 per cent) of sudan III in 70 per cent alcohol filtered and stored in a tightly stoppered bottle keeps a year or more. When testing for fats care must be taken to prevent evaporation of the alcohol with consequent precipitation of the dye.

Mount sections on a slip in water and examine. Draw off the water by means of strips of filter paper, replacing with 70 per cent alcohol. Then add a drop of the sudan III solution. After 15 to 20 minutes, draw off the dye, adding 70 per cent alcohol at the opposite side of the cover glass. Draw through, adding more alcohol to remove all of the excess dye. Then add a drop of glycerin or of levulose jelly (8 gm. levulose in 10 c.c. water) diluted with a drop of water to hasten the flow. Fats and fatty oils will be stained red; also suberin and cutin membranes, and ethereal oils and resins will be red if they are present. For permanent mounts levulose jelly is rather better than glycerin, since it clears better and hardens quickly.

Sudan IV (Scarlet red). This dye, like sudan III, is insoluble in water, soluble in alcohol, and more soluble in fats, oils, and resins and in suberized and cutinized membranes. Since these dyes do not form salts (Michaelis*), they are ideal fat stains, especially for plant tissues where the cells contain many different substances.

A concentrated solution in 70 per cent alcohol, filtered and stored in a tightly stoppered bottle, keeps for many months. Ten to 15 c.c. for daily use should be kept in a 15 c.c. bottle having a ground-in pipette with rubber nipple. If the pipette is always replaced immediately after use, to prevent evaporation of the alcohol, the dye does not precipitate out.

When only minute droplets of fat are present in the cells, stain with scarlet red. After ten to fifteen minutes, remove all of the excess dye by drawing it off with filter paper, replacing with 70 per cent alcohol, until the filter paper shows no slightest trace of color. Then add a drop of concentrated H_2SO_4. The red droplets flow together becoming blue as the dye dissolves in the acid (Tunmann).

Michaelis' method is better since it does not involve the use of strong acid: After staining with scarlet red and removing all of the alcoholic dye, as before, add water, drawing off the alcohol. The scarlet red which

* Michaelis, L., Fett. in Krause, R. Enzyklopädie der Mikroskopischen Technik, Ed. 3, i, Berlin, 1926.

was dissolved in the fat droplets is precipitated out in tiny clusters of red needle crystals.

Other dyes which are soluble in fats, oils, resins, and in suberin and cutin membranes are sometimes used advantageously. All are made up in concentrated solution in 70 per cent alcohol.

Alkanin (deep red) is a good fat stain. The chief objection is the long time required, four to sixteen hours. There seems to be a slow penetration into the cells as well as slow solution in the fats.

Indophenol as a free base stains fats blue. Its salts do not stain fats (Michaelis).

Oil red A (Nat. Aniline & Chem. Co.) stains fats yellowish red. It is a rapid stain but the sections do not clear as well as after the use of sudan III or IV.

Dimethylamidoazobenzol as a free base dissolves in fats giving them a clear yellow color, but it forms red salts with acids. The salts are not soluble in fats. As a fat stain it is satisfactory only with tissues containing neutral fats. In the case of acid tissues the cell contents are likely to be pink while the oil droplets may be faintly yellow or unstained.

Osmic Acid. Osmium tetraoxide is reduced to black metallic osmium by oleic acid, olein, mixed fats containing oleic acid, ethereal oils, resins, lecithin, and tannins. These substances are, in turn, blackened or browned by the precipitated osmium. The saturated fats and fatty acids do not reduce osmic acid. Thus the reaction is of limited usefulness as a fat test.

Examine sections in water on a slide for highly refractive drops. Draw off some of the water, adding a drop of 1 per cent osmic acid. Note blackening. The nature of the blackened or browned substance must be determined by other means.

3. Saponification. This specific reaction should be made always in addition to the preliminary solubility and staining reactions. The best saponification fluid for the identification of fats in cells is made up of equal volumes of concentrated water, KOH and 20 per cent ammonium solution (Molisch), stored in a brown bottle. It is well to make up a fresh solution every six months, since it deteriorates on long standing.

Put sections on a slip in a few drops of the saponification fluid and cover, avoiding the inclusion of air-bubbles. Ring the cover glass with wax to prevent the formation of carbonates at the edge. Since from three to five days are required for complete saponification, it is best to keep the slide in a moist chamber. Observe the progress of saponification at intervals up to the fifth day, when it should be complete.

Usually in sixteen to twenty-four hours fine needle crystals of the potassium salt of the fatty acid appear at the periphery of some of the oil drops. Oil drops at the edge of the section show the beginning of saponification before those within the cells. After two to three days most of the fat drops will have the needle crystals at the periphery. A few drops may be

completely saponified, leaving a group of the soap crystals. Some fat drops, without change of form, become doubly refractive, indicating that the fat is partially saponified. This type of partial saponification occurs more frequently if the strength of the saponification fluid is decreased, by aging or by dilution. By the fourth or the fifth day every fat drop should be completely saponified.

Tartaric acid and some alkaloids form crystals with potassium hydroxide, but they are readily differentiated from the potassium-fatty acid crystals. The potassium-alkaloid crystals are formed quickly, usually within 30 minutes; and they are in scattered clusters, not on the surface of droplets. Potassium tartrate does not form needle crystals.

4. **Myelin Formation.** This specific reaction is especially valuable for rapid identification of fat globules. Essentially it is partial saponification with the formation of doubly refractive drops.

Observe sections on a slide in 10 per cent ammonia or in 10 per cent KOH. Usually within 30 minutes the contour of fat drops changes and little protuberances appear, which may enlarge somewhat. Gradually the drops become doubly refractive.

The remarkable myelin forms frequently pictured occur with the fats of comparatively few plants but all fats and oils show the early change of the globules from spherical to an irregular form. (Sophia Eckerson).

C. SPECIAL METHODS FOR PARTICULAR PLANT GROUPS

It was advantageous to treat the first portion of the present chapter from the viewpoint of one plant group. The angiosperms were selected as offering the widest range of methods, and the most familiar illustrations. Therefore it is not necessary to discuss them much further in this second portion. In the degree to which the other plant groups differ in size and texture special methods are needed, and these are suggested here, with references back to the first portion. The bacteria have been handled separately (Conn, Chap. III).

XIII. Algae

In general the methods recommended in the more general sections for the preservation and preparation of filamentous specimens are applicable to algae, so that careful attention to the fixing and staining sections will be sufficient to give a working basis for the start of an algal study involving the development of a precise technique. Therefore little attempt will be made to present here a complete description of methods, rather emphasizing the special aspects of a few peculiar adaptations.

1. **For herbarium specimens** and general morphological studies many filamentous types are preserved quite sufficiently well by simply drying on clean glass slips or freshly split mica. These preparations, if made of marine organisms at the seashore, may remain moist, due to the hygroscopic

properties of the salt, and so should be dried in the sun or a desiccator and kept carefully wrapped in a moisture-proof container until they can be filed under dry atmospheric conditions. When desired for use they may then be moistened with water, covered and studied with ease and without disturbing the specimen, and dried again for storage. Care should be exercised in reporting cell measurements based on such material. It may be better to moisten with carbol-glycerin or lactic-glycerin (p. 111), but this must be well washed off if the material is to be again dried. (For the handling of herbarium material dried on paper see p. 110.) Algae which adhere as close films to the paper are sometimes difficult to remove. With a keen knife cut lightly through the surface of the paper around the margin of the piece needed. Then cover with a drop of water, let it stand for a time, and finally gently scrape loose the desired portion of alga with scalpel and needle, transferring to a drop of lactic-glycerin on a clean slip, where further dissection may be accomplished and the drop covered for study or preservation. Eosin may be used to stain such material before placing in lactic-glycerin.

2. **Properly fixed algal material** is most generally secured by a suitable modification of Flemming's fluid applied effectively, since this fluid may be expected to give the best results. It is usually well to start with a weak formula, at least on the filamentous types. Marine forms are, of course, fixed in solutions made up with sea water. Chrom-acetic mixtures do well for general habit and grosser cell structure, while formalin-acetic-alcohol and Keefe's formula are good for preserving habit material (p. 139). It is only common sense to avoid drying material on the slide, either before or after fixing, for any but crude morphological studies. However, drying may be all right for observations on flagellae (p. 146), and does no great harm for observations on the position of nucleus and pyrenoid in small unicells, but cannot serve for observations on chromatophore structure, since the chromatophores readily shrink. The fixed specimens of filamentous or unicellular types are washed, stained carefully in Heidenhain's hematoxylin, safranin or magdala red, and counterstained if desired with erythrosin, orange G, aniline blue, cyanin or light green (p. 132). Then they are dehydrated (pp. 11, 115) cleared (p. 115) infiltrated in balsam (p. 143) or Venetian turpentine (p. 143) and mounted. The filamentous types may also be imbedded and sectioned for nuclear details, the proceeding being gradual (p. 159). The mucus so often present about algae frequently interferes with infiltration and imbedding. Dilute aqueous methylene blue will stain the cell walls of algae, particularly fresh Phaeophyceae, very readily.

3. **Epiphytic and endophytic types,** if small, are best fixed, stained, and mounted in situ, or only removed just before mounting. For teaching purposes it is often well to mount on a slip both light and dark stained samples, since chromatophores show best at quite a different degree of

differentiation from that suitable for nuclei and pyrenoids. Mats of filaments (as *Vaucheria*) can usually be best prepared undisturbed and later teased or cut apart just before mounting, and crusts of filamentous or unicellular types or sticks or stones can be scraped off and dissociated likewise just before mounting. (For handling sporelings or species cultured on slides see p. 172.)

4. **Myxophyceae** when collected may be badly mixed with other forms. *Oscillatoria* and hormogonia of other genera will creep out from such a mass if it is placed on a wet slip in a damp chamber, and will spread over it, where they may be dried, or fixed in some cases by inverting the slip flatly on the surface of a suitable fixing fluid. Dried mounts preserve the cell organization more effectively here than with most algae, but are quite unreliable from the cytological standpoint. Many species of the Stigonemaceae show splendid intercellular connections. Accurate fixation through the sheath frequently present is probably impracticable with present methods; indeed, critical inspection of the results of cytological studies on Myxophyceae suggests that little, if any, adequate fixation has been accomplished in the group. Staining with Delafield's hematoxylin is reported to give a polychrome result, some granules being blue and others pink in tendency. While the writer has seen no such specificity, it does give a good general stain. Heidenhain's hematoxylin followed by erythrosin gives good contrast. Safranin stains mature spores well and can be followed by aniline blue, light green, crystal violet, or Delafield's hematoxylin. Methyl green stains young heterocysts readily.

5. **Chlorophyceae** of the motile groups are handled in general like independent flagellate protozoa (p. 412). Fixation is excellent after Flemming's fluid, but hot sublimate-acetic may serve well for Volvocales. The sedimentation method of handling, even to the use of a centrifuge, is preferably used for passing the organisms through the fluids. Dehydration by the concentration of glycerin is easiest and best. The sample should be agitated as little as possible to avoid clumping and the breaking of the flagellae. They may be stained and mounted entire as strewn mounts, using the balsam or the Venetian turpentine infiltration methods, or they may be sectioned after imbedding in paraffin.

Desmids, if of the filamentous genera, are handled like *Spirogyra*. Unicellular forms, when in great abundance, are handled by sedimentation. If rare they are picked out with a fine pipette, placed in a small drop of water on a slip which is inverted over the mouth of an osmic acid bottle, where the vapor fixes and darkens the cell, which is flooded with 10 per cent glycerin that is allowed to concentrate, and later replaced, if desired, with glycerin jelly for mounting. The sheath of desmids, both filamentous and unicellular, can be effectively stained by a method proposed by the writer:* "Fresh living material is placed in a .05 per cent aqueous solution

*Taylor, W. R. *Trans. Am. Micros. Soc.*, 1921, xl, 94.

of methylene blue for forty-five to sixty seconds. It is then removed, rinsed in distilled water and placed in a $\frac{1}{10}$ saturated aqueous solution of picric acid. This serves to fix the stain and brings out in a most striking manner the striations in the sheath. The material may be examined in the picric acid solution, or removed after a minute or two to water. Preparations are best used soon after staining, as the sheath begins to disintegrate after a few hours." This is an especially favorable method for Placcodermae, such as *Hyalotheca*, whose sheath shows very well the radiations resulting from its method of formation.

Chlorophyceae of the larger filamentous groups are handled after the general system for filamentous organisms, being especially careful to avoid shrinkage of the protoplast. Chamberlain* recommends keeping the chromic acid up to 1 per cent and increasing the acetic acid until shrinkage is overcome, as he finds that algae require more acetic acid than do higher plants. Flemming's fluids are generally better than chrom-acetic, though differentiation of stains may be somewhat more difficult. *Spirogyra* gives especially splendid mounts when carefully handled. The fixed filaments are gently washed, stained in Heidenhain's hematoxylin with care to avoid overbleaching in differentiating, well washed and placed in 2.5 to 5 per cent glycerin. Here they are dehydrated by concentration, the process occupying two to three days to a week. It should not be unduly rushed, and must be complete. Nucleolus, chromosomes and pyrenoids should be black, chromatophores steel gray, and general cytoplasm pearl gray. The contour of the chromatophore margin should show the characteristics of the species used. No counterstain should be necessary. After dehydration the balsam infiltration method (p. 143) should be followed. Conjugating material is somewhat more sensitive than sterile filaments, especially to the xylol-balsam change. Filaments with zygospores are most effectively stained with safranin-aniline blue, the spores being bright red (p. 133). Massive Chlorophyceae (Codiaceae) offer no special problem, for pieces of tissue may be handled like pieces of soft tissue from higher plants. If calcareous they may be hard to fix, and must be decalcified (p. 112).

Charales are generally so sturdy that they should be handled like delicate parts of flowering plants for imbedding and sectioning. Care should be used in orienting the material for cutting, since from inappropriate angles the sections do not show the geometrical regularity of division planes which characterize the organs of the group. Apical cells are frequently needed for demonstration. Tips of main axes from sturdy types should be selected and the branches and leaves trimmed close to the tip. Fixation in Flemming followed by imbedding, sectioning and staining in safranin and Delafield's hematoxylin is best. In addition to the apical cell there should show young stages of leaves, branches, and on fertile plants, of reproductive organs. For older stages of reproductive organs cut off

* Chamberlain, C. J. Methods in Plant Histology, Chicago, 1924, p. 176.

branches of appropriate age and imbed individually. Sections of the branch are best cut longitudinally and axially through the reproductive organs. The branch may show a range of developmental stages. Mature antheridia may be punctured and stained in toto, and then dissected apart when ready to mount. Mature oögonia are extremely hard to cut. For habit mounts the more slender species are to be preferred, and they may be handled like filamentous algae, mounting under sufficient pressure to flatten out the specimen. Safranin and aniline blue will generally give a brilliant stain.

6. Heterokontae are not well enough known or widely enough studied to have developed special technique. In general they are handled like Chlorophyceae of similar form. The wall seems to be often more resistant to fixing and staining fluids. The peculiar wall structure, each cell having two overlapping portions, equal in size or like a lid on a cylinder, is emphasized by swelling with strong caustic potash and staining with Congo red, or by separating the portions by treatment with 30 per cent aqueous chromic acid, cold or with the aid of heat.

7. Bacillarieae (**Diatoms**) because of their peculiar silicified cell wall offer special problems in treatment for systematic study. Often the living cell content is a positive disadvantage, and must be removed before the species can be studied. If the living cell contents are to be observed they are prepared like desmids or other unicellular Chlorophyceae. Care is needed in destaining, for penetration of fluids through the wall is irregular. Material (after hematoxylin) should be removed from the iron-alum a little before it seems completely ready, so that it will not become unduly destained before the iron-alum can be washed out. The material should be dehydrated through glycerin and infiltrated with balsam. If the diatoms are to be studied from the systematic standpoint it is generally the silicified wall alone that is needed. A great number of methods of cleaning material have been devised, some of limited and others of wide application. For recent or living material one general system will suffice.

The mass should be allowed to settle and the supernatant water poured off. An equal volume of concentrated hydrochloric acid is then poured on, mixed, and gently boiled. After a few minutes action will have been completed and the flask or test-tube is allowed to stand and the material to settle. A brief washing in 2 or 3 changes of water follows, and then the water is poured off and an equal volume of nitric acid poured on and similarly boiled, followed in turn by washing and boiling in sulphuric acid. This last will usually cause darkening of the material through carbonization of any remaining organic matter. To clear this up crystals of potassium chlorate should be cautiously added one at a time to the hot acid, protecting carefully one's eyes and neighbors. The use of a chemical hood through all this process is helpful because of the unpleasant fumes. Each crystal of potassium chlorate will probably cause a small explosion and the formation of chlorine gas, which with the oxygen will effect bleaching and solution of the remaining organic matter. If a large quantity of material is being handled it may be necessary to repeat each stage of the process before passing on to the next. The final washings must be

thorough. The diatoms are then separated from the sand by fractional sedimentation, the coarse sand going down first, then coarser grit and large diatoms, and finally small diatoms and fine clay particles. It is possible practically to free any desired sample from extraneous matter by judicious and repeated sedimentation. Or, the sample may be placed in a small jar with a large quantity of water and the jar revolved between the hands, when the material will rise in the center of the jar and the particles become distributed according to their relative densities. After washing, material is best stored in small bottles with ample 50 per cent alcohol. In mounting it is necessary to use absolutely chemically clean cover glasses (p. 32), if the job is to be neat and the distribution of the frustules uniform. It is best to let the material in the bottle settle and replace the alcohol with distilled water. Shake and put a drop of material on each cover, where it should spread out evenly. After the diatoms have settled in the bottle replace the water again with alcohol for storage. Allow the covers to dry thoroughly without disturbing them, and then heat to drive off any water in the frustules or their pores. These are spoken of as strewn mounts. Add a drop of thin xylol balsam and heat. Then invert on a drop of balsam on a slip and warm until firm. Bubbles of gas formed by heating the balsam solvent will disappear in time, as will small air bubbles. The use of special highly refractive mounting materials recommended for diatoms is rather a special topic and reference may be made to older works on microscopy if difficulties are encountered. Many species show their more delicate markings best if mounted dry. "Cells" should be prepared by making several superposed rings of cement (p. 140) on a clean slip. Covers with dried frustules are prepared as above, and inverted while quite warm so that their edges lie on the cement rings. Press each cover slightly into the ring so that it adheres. Let it cool and give a coat of rather thick cement. If well pressed into the ring no difficulty should be met from cement running under the cover.

Selected diatoms are generally isolated from a mass collection and mounted separately for reference. Strewn covers are prepared as usual and fastened, material up, on slips. A microscope equipped with a mechanical finger is used. A ring above the objective (about 8 to 16 mm. focal length) bears a lever or rod which can be raised and lowered by a screw, and pushed toward or from the optical axis through a smooth-fitting sleeve. A fine glass filament is cemented with wax at the tip of the rod. The free tip of this is moistened, usually with a very faint trace of oil or grease to make it slightly sticky. It is then adjusted to the center of the field of view and raised up out of focus by the screw. The objective is focussed on the slide and the diatom desired is selected. Then the filament of glass is lowered into contact with the diatom, which should, with a little manipulation, adhere lightly to it. With the coarse adjustment the objective and specimen are raised together under observation, and the strewn mount removed from the stage. Another slip should be ready bearing a cover in the center of which is a thin smear of gelatin dissolved in glacial acetic acid. This is slipped into place and the diatom lowered into contact, still under observation. By gently breathing on the slide, moisture causes the diatom to stick to the gelatin. On drying, the cover may be mounted as usual without disturbing the specimen, and its position marked by spinning rings of colored ink or cement about it as a center. By manipulation of the mechanical finger diatoms may be placed in any desired position, to show face, girdle or end views, and collections may be selected and arranged on a single slip, to several hundred in number, to exemplify the flora of a given sample, to give test plates with forms suitable for testing a wide range of microscope objectives, or even only to demonstrate the marvelous beauty of the diatoms themselves.

Diatoms are not usually abundant in highly calcareous collections, but on tropical shores are usually admixed with a predominating bulk of foraminifera, calcareous sand and shells. Consequently a quantity of the

original material may be greatly concentrated by gradual addition of hydrochloric acid to the sample till effervescence ceases, followed by sedimentation or centrifuging and the usual treatment. Plankton types and many bottom forms, especially of the tropics, are often so delicate that the severe general schedule offered above will destroy the markings or even the entire frustules. For these the sulphuric acid may best be omitted, and the other treatments curtailed or performed with weaker acids. It may be possible to do no more than dry the sample on a slip without removal of the organic matter. Many coarse types are adequately prepared if a sample of the original material is spread on a cover, which is then placed material side up on a copper plate and this heated dull red or until the organic matter is burned away. This can generally be accomplished without softening the cover, which is mounted as usual.

Fossil diatoms in diatomaceous earths or soft diatomaceous rocks are generally free from much organic or calcareous matter. The sample is broken to fine granules and boiled in hydrochloric acid until the frustules may be separated by shaking. These are then separated by fractional sedimentation. Usually there is a great proportion of broken shells and these come away with the small species. Diatoms in marl are accompanied by clay and may be difficult to separate from extraneous matter. Diatoms in harder rocks are best shown by grinding thin sections after the fashion of other fossil samples (p. 122).

8. Phaeophyceae in general offer few peculiarities requiring special instructions. In general they can be handled like green algae of similar texture and form. The walls of filamentous types (as some species of *Ectocarpus*) are often quite horny in texture when imbedded and do not cut cleanly. They are best studied as whole mounts if possible, and are well stained in the fresh state by methylene blue. *Cladostephus* and its relatives show spendid asters at the division stages of the apical cell, and longitudinal sections of selected apices give good material for their study (p. 147). The soft, gelatinous walls of the fleshy browns swell and shrink greatly as they are wet or dry, and so material imbedded for sectioning in paraffin or celloidin generally becomes much distorted and sections readily work loose from the slides. The freezing method (p. 125) is advantageous with such types. In some species with dilute gelatin between the cells or filaments prolonged preservation in formaldehyde (which probably becomes quite acid) dissolves the gelatin so that the specimens imbed and cut perfectly in celloidin. Especially fine preparations exhibiting the structure and reproduction of the Chordariaceae may thus be made from *Castagnea* that has been stored for a couple of years. *Fucus* as the commonest laboratory type must frequently be attacked for the preparation of routine teaching slides. Flemming's fluid is the best fixing agent, and the material, in vigorous health, should be cut into the small pieces that are to be sectioned before fixation is attempted, in order that the gelatinous walls and the slime may

interfere as little as possible with its action. For the growing tip the apices are cut from sterile branches as pieces 2 or 3 mm. square, fixed and sectioned in paraffin in the plane of the branch. The structure of the branch is best shown in fresh transverse sections mounted in water. For the reproductive organs it is best to cut the receptacles lengthwise and then cut out small blocks with a few conceptacles, fix, imbed and cut in paraffin as transverse sections, selecting as best those that pass through the pore of the conceptacle. Heidenhain's hematoxylin followed by orange G or erythrosin gives the best stain, but Delafield's hematoxylin is good enough for material not Flemming-fixed. Eggs of *Fucus* may be fertilized and fixed at chosen intervals after scraping from the dish containing them, and to which they become more or less attached. By imbedding in paraffin sections may be secured showing the entrance of the sperm, fertilization and the first mitosis with the asters and other characteristic features. Sporelings of *Fucus* are easily mounted and instructive, and sporelings of other Phaeophyceae (especially Laminariaceae) are similarly valuable, prepared by the general method (p. 172).

9. Rhodophyceae offer considerable difficulty from the standpoint of technical practice. Unicellular types (as *Porphyridium*) are handled as smear preparations on clean covers. Filamentous types are fixed in masses and handled like the filamentous greens. Flemming's fluid is by far the best fixing agent. Some form of the weak formula should be used, and not allowed to act very long: a few minutes to an hour or so. The filaments disintegrate rather readily in dilute acids. It is best to wash while dehydrating slowly through the alcohols, using close stages for short periods and keeping the container in a dark place. Imbedding and sectioning, or staining and infiltration with balsam, follow the usual methods for filamentous Chlorophyceae. The large coenocytes of some genera (*Griffithsia*) call for great care in clearing. Safranin followed by aniline blue gives excellent contrast. Nuclei will generally be purple, chromatophores light blue and cell walls pink. Heidenhain's hematoxylin is dependable for accurate details. For morphological studies chrom-acetic or formol-acetic-alcohol is suitable, followed by preservation in 70 per cent alcohol. Fixation and preservation in formaldehyde is not recommended for filamentous types, since these tend to disarticulate as the formaldehyde becomes acid. It is possible that non-acid formaldehyde (p. 112) would do perfectly well. Fleshy species are best sectioned by the freezing method (p. 125), or imbedding in soap (p. 125). Calcareous species are very difficult from the cytological standpoint. Probably the best results are to be secured by fixing such genera as *Melobesia* and *Corallina* in a relatively large volume of Flemming's fluid and removing the CO_2 rapidly by the aid of a vacuum pump, adding additional chrom-acetic solution as the original becomes exhausted. Massive types such as *Lithothamnion* and *Goniolithon* are probably impracticable cytologically. For histological and morphological studies of these genera

chrom-acetic decalcification and fixation is adequate, and imbedding in paraffin best for cutting. As there is little tissue differentiation Delafield's hematoxylin alone is a good stain. In addition to these customary ways of preparing material it is often desirable to swell up developing procarps and cystocarps and to cause the cells to stand apart from each other. This is especially effective on *Griffithsia* (Lewis). Living material is fixed with chrom-acetic or with aqueous iodine and placed in 10 per cent glycerin. Here the walls of the procarps and cystocarps gelatinize and swell, so that the intercellular connections may be readily recognized. A trace of eosin in the glycerin will help by tinting the specimen. To trace the development of the cystocarp in those Cryptonemiales with long oöblastema filaments is not so difficult if the species preserve a distinctly filamentous structure and are soft. In the firmer types it is often difficult. *Agardhiella* will serve as a good example for demonstrations, being widely available. Select sturdy tips and split them lengthwise with a sharp razor, being careful to go exactly through the apex for best results. Lay both halves split side down upon the cover glass, and then invert upon a slip and add water from the margin. The carpogenic branches and auxiliary cells with trichogynes and oöblastema filaments can be seen upon the big cells of the medulla wall, having slightly denser contents, which can be tinted with eosin.

10. **Sporelings and epiphytes** are effectively cultured on slides and stained and mounted in position. Smear mounts, bacterial zoöglea spreads, and other fungal mounts involving parts of, or entire, colonies, are familiar in method (pp. 175, 90), but for unicellular or attached filamentous algae, early stages of bryophyte or algal sporelings, etc., it is convenient to fix, stain and mount on the original substratum. The slips used should be absolutely clean. If the organisms seem to have difficulty in making firm attachment to the slips these should be etched on one side with hydrofluoric acid, ground with a sand blast, or given a matte grain by rubbing together with emery powder. When handling positively phototropic organisms (such as zoospores from *Stigeoclonium*, *Ulothrix*, *Chaetophora*, *Ulva*, *Ulothrix* or *Oedogonium*) the slips should be placed in the container with the material, and between it and the light source. For spores of Rhodophyceae, or other non-motile spores, and eggs of the Fucaceae, the slips should be distributed over the bottom of the container. In any case as soon as the desired material has become attached, the slides should be removed to a clean container with fresh (preferably filtered) water, and handled by methods appropriate to the particular organisms under observation. At convenient intervals slides may be removed, placed in staining wells of fixing fluid and handled throughout as in the smear method (p. 116). Often highly interesting material (like *Coleochaete*) will form a green coating on the walls of aquaria, and if slides are placed in contact with the walls a good growth will appear on them equally readily. Instructive mounts of small diatoms are a frequent by-product of this method. The fixing fluids

and stains to be used are those ordinarily adaptable to whole mounts of filamentous algae (p. 165). For sporelings of Fucales simple dilute Delafield's hematoxylin seems best.

XIV. Fungi

The fungi are handled similarly to algae in many ways, for both are unicellular to filamentous types of organisms which attain rigidity and bulk in more complex groups by lateral association and specialization of the filaments. But the cell wall is of different material and texture, and chromatophores are absent. The fungi are, furthermore, often parasitic, and the nature of the host's tissues may determine the character of the technique even more than the parasite. Minute forms, if in great quantity, may be handled in bulk through fixing, staining and dehydrating. Yeast, for instance, may be managed in this way. Cultures of small forms, including bacteria (see Chap. III) and germinating spores may be spread on slips, dried, fixed by passing a few times quickly through a flame, and then stained. Or they (cultures, or spores) may be spread on slips which have been wiped with a little Mayer's albumen and are allowed nearly to dry, and then inverted flatly upon the fixing fluid, which may conveniently be in a Petri dish with some fragments of glass rod to keep the slides from coming into contact with the bottom. After fixation they may be washed and treated as sections. A method advocated by Bachmann* may give good service:

> Prepare some thoroughly clean sterile slips. Onto one pour the culture medium (with gelatin or agar base) inoculated with a suspension of the organisms to be investigated. Place another slip upon the first and draw apart, so as to leave quite a thin film. The slides should then be incubated under sterile conditions in a damp chamber (as a Petri dish with wet filter paper on top and bottom). When the colonies are ready the slides may be fixed and handled precisely as if bearing smears or sections. For yeast she recommends potato broth agar, which, like all media for this method, must be cleared. Heidenhain's hematoxylin followed by a counterstain (if desired) and mounting in balsam is usual. Or yeast may be cultured in fluid and killed in bulk in a saturated aqueous solution of corrosive sublimate, where it may be left twelve hours, following with thorough washing in water, 30 per cent and 70 per cent alcohol and finally absolute methyl alcohol. A drop of a suspension of the cells should be placed on a coverglass and allowed nearly to dry. The cover is then flooded with water, and the yeast allowed to settle. Then the water is drained away and the cover dried. After thorough drying, which should cause the yeast to adhere, the cover may be wet and stained with the combined fuchsin-methyl green mixture and mounted in damar balsam,† or Flemming's solution may be used, followed by Heidenhain's hematoxylin, then glycerin dehydration (p. 114) and infiltration in balsam (p. 143).

Many species of fungi, especially from culture on solid media, are in the form of a loose mass of mycelium. For direct examination it is usually necessary to remove the air from between the filaments in mounting, and

* Bachmann, F. M. *Amer. J. Botany*, 1918, v, 32.

† Chamberlain, C. J. Methods in Plant Histology, Chicago, 1924, p. 196.

this may be accomplished by flooding the material on the slide with 70 per cent alcohol. The material may be mounted for examination in 3 per cent acetic acid, 3 per cent potassium hydroxide or weak chloral hydrate. The potash is especially good if the sample has been dried. Aqueous media of a low refractive index are best, not lactic-glycerin and the like. For general fixation absolute alcohol, saturated corrosive sublimate with 1 per cent acetic acid in 95 per cent alcohol, or Gilson's fluid are to be recommended. Duggar* recommends for fungi a modified Gilson's fluid with the following composition:

Alcohol, 95 per cent, 30 c.c., distilled water 270 c.c., glacial acetic acid 2 c.c., nitric acid 5 c.c., mercuric chloride 10 gm. The usual procedure after a mercurial fluid should be followed (p. 158).

He also gives a special Flemming's type fluid: 10 per cent chromic acid 1.5 c.c., 10 per cent acetic acid 1.0 c.c., 2 per cent osmic acid in 2 per cent chromic acid 5 c.c., distilled water 37.5 c.c. He prefers to bleach the material in bulk in 95 per cent alcohol, 75 c.c., hydrogen peroxide 25 c.c. before imbedding.

Material of fungi is essentially exceedingly delicate, at least in the filamentous types, and offers considerable difficulty in handling through the alcohol changes and in imbedding. For transferring Duggar recommends little dipper-shaped wire gauze ladles, transferring the ladle with its contents from stage to stage without disturbing it. Material to be examined on the slide without extended treatment may be stained most readily with 0.5 per cent aqueous eosin, or with alum-eosin (0.5 per cent of each). Filaments hard to stain take most readily to Ziehl's carbol-fuchsin as prepared for bacteria. For material sectioned for histological or cytological studies the ordinary methods may be tried. Stages showing brightly colored spores (*Xylaria* ascospores, *Puccinia* teleospores) may be satisfactorily differentiated merely by the use of such a counterstain as light green or Delafield's hematoxylin. *Xylaria* and other similarly tough forms may sometimes be cut satisfactorily unimbedded like stems of vascular plants. Apothecia of *Peziza* and genera of similar texture, including some Lichens, may be broken up into small pieces, stained in bulk in aqueous eosin, rinsed, transferred to 2 per cent acetic acid and there teased out to show the developmental stages. Or the small pieces may be stained in eosin, dehydrated and cleared with clove oil, and teased out before mounting in balsam. For sectioning, old apothecia may be selected, imbedded in paraffin and sectioned rather thick, when the spores take safranin stain well, and light green or Delafield's hematoxylin may be used as counterstains. For the maturation divisions, including demonstrations of centrosomes and intranuclear mitosis, exceedingly small specimens must be used, and the sections cut thin and stained with Heidenhain's hematoxylin. The differentiation of fungus mycelium from host tissue in the case of pathological infestation generally offers difficulty. The usual histological combinations may be tried, and may give satisfactory results, but frequently fail.

* Duggar, B. M. Fungous Diseases of Plants. Boston, 1909.

Vaughan* recommends the following procedure: A stain combination known as Pianese IIIb is prepared by mixing malachite green 0.50 gm., acid fuchsin 0.10 gm., martius yellow 0.01 gm., distilled water 150.0 c.c., 95 per cent ethyl alcohol 50.0 c.c. The tissue sections are washed in water or alcohol and stained in the mixture fifteen to forty-five minutes. The excess stain is removed with water and decolorized to differentiation with 95 per cent alcohol containing a few drops of hydrochloric acid. The material is then cleared with carbol-turpentine (carbol-xylol would do, probably) washed with xylol and mounted in balsam. He has also modified the method for the staining of germinating spores on the epidermis of infected plants. The leaf is prepared, a drop of spore suspension placed on the desired region and the leaf kept under suitable cultural conditions. At the proper time the test area is cut out, fixed for twenty-four to thirty-six hours in equal parts of glacial acetic acid and 95 per cent alcohol, washed in 50 to 70 per cent alcohol, stained for fifteen to thirty minutes in Pianese IIIb, washed in water for two minutes, very hastily run through acid alcohol, dehydrated and cleared as above.

The mycorrhiza found in *Corallorrhiza* rhizomes and in the roots of epiphytic orchids frequently gives some difficulty in staining. The writer has had best success after fixing in chrom-acetic, and staining sliding microtome sections heavily with safranin, destaining, and counterstaining in succession with methyl (or light) green and orange G. The older mycelium and host nuclei should be deep scarlet, the younger pinkish or green, the host walls yellowish or if lignified, red. Lichens give considerable difficulty in preparing sections of imbedded material, and may best be studied from sections of fresh or fluid preserved material cut unimbedded, though paraffin imbedding may be tried and will at times succeed perfectly. Heidenhain's hematoxylin followed by erythrosin, or cyanin followed by erythrosin make good stains. Myxomycetes in the plasmodial state may be fixed excellently with Flemming's fluid, and if the plasmodium has been induced to climb onto a slip the flowing mass may be fixed as a whole mount. Imbedding in paraffin is all right if the material does not have to be kept with the substratum after fixing, but if it surrounds leaves of grass or decayed sticks it is better to use celloidin. The stages in the formation of the sporangia and the segmentation into spores and capillitium require very thin sections, and Heidenhain's hematoxylin is the only stain precise enough to use for nuclear features. In fact, throughout the fungi, this stain is the only one generally applicable to the study of nuclear details because of the very small size of the structures involved.

Spores of fungi and other plants may be caused to adhere to the slips upon which they are to be studied if these are first smeared with a film of Mayer's albumen before the spores are spread on the surface. The slide should be inverted upon a suitable fixing fluid, or dried and heated, or inverted upon alcohol, to coagulate the albumen. After this, the technique to be followed is similar to that adopted for bacteria (p. 91) or smears of pollen grains (p. 116). Most often they are simply mixed with balsam or glycerin jelly and covered.

* Vaughan, R. E. *Ann. Missouri Bot. Gard.*, 1914, i, 241.

XV. Bryophytes

The Bryophyta present, in general, few special problems. Preservation for general morphological purposes may be effected in Keefe's fluid, or in formol-acetic-alcohol, or formalin with copper acetate (p. 139). Habit mounts of whole plants, and morphological studies of thallus form, leaf form and cell arrangement, spores and elaters, peristomes, etc., are most readily mounted unstained in glycerin jelly. As they often make thick mounts the use of melted resin-lanolin for sealing is recommended (p. 140). In no group of plants is the use of free-hand sections more essential to the quick determination of species or interpretation of anatomical features (p. 123). Peristomes, calyptras, etc., for whole mounts in balsam should be dissected out and dried between two slips under light pressure. It is well to moisten with xylol before adding balsam to drive out air, which it may be difficult in any case to remove from the capsule and annular cells, though boiling in balsam may help. Cytological studies can be best made on material fixed with Flemming's fluid, or the special fluids for mitochondria, etc. (p. 198). The cell walls of mosses often become brittle if the material is imbedded in paraffin and cut poorly, so celloidin is often preferable. Picric acid fixing formulas are helpful here, following with hematoxylin stains. Fleshy, unchambered Hepaticae may be difficult to infiltrate in celloidin and do best in paraffin, especially for apical or chambered cells, (Ricciaceae and Marchantiaceae) which are best shown in vertical median longitudinal sections. For the morphology of Ricciales thalli celloidin is best if cut fairly thick. Sexual organs do very well in paraffin. Sporangia are less readily cut as they become older, and should be punctured or cut open so that the reagents may penetrate. If old and horny it may not be possible to cut sections even in celloidin, and it may be necessary to treat the material like hard woody tissue (p. 128).

XVI. Pteridophytes

For general preservation, cytological fixation, histology of the vegetative organs, etc., the general methods are quite efficient, since the types of tissue present approximate those of phanerogams, for the preparation of which the more general sections were designed. The leaves of some Lycopodiales will give trouble in cutting if paraffin-imbedded. This extends to the comparatively young strobili, which often give trouble, and particularly in old stages where the sporangia and spore walls become hardened. The strobili should be trimmed flat from opposite sides to facilitate penetration of reagents. Axial sections are usually to be preferred. *Equisetum* stems present the difficulty of silica impregnation, and must be desilicified (p. 113) and imbedded in celloidin for really good sections. The growing apex will cut well in paraffin without special preparation. Filicales offer several points worth notice and caution. Root tip sections for apical cells

are best cut absolutely median and longitudinal, so that the segments may show to best advantage. For these, if cytological features are not desired, chrom-acetic fixation followed by Delafield's hematoxylin is a most efficient, simple technique. Stem tips may be similarly treated, trimming away the tough ramental scales. Older roots and stems are handled by the general methods for firm or woody tissues. Leaves may be sectioned fresh, or for more accurate details imbedded. Old sporangia with thickened annulus will give some trouble in paraffin sections, but young stages cut perfectly well. For classroom preparations median sections through the stalk of the indusium give most diagrammatic results. For cytological purposes it is well to watch that penetration be assisted in all possible ways, since the sporangial coats, especially of the Eusporangiatae, are rather resistant to fixing fluids (p. 155). Whole mounts of leaves, especially of the lobes of thin, large leaves with sporangia, are very satisfactory for showing the structure of the sorus. Clear the leaf by partial maceration in caustic potash, wash and stain in safranin. Destain cautiously and counterstain with methyl or light green; differentiate, dehydrate and clear carefully and mount in balsam. The counterstain should be rather dilute. Old indusia, annulus and mature spores will stain red, as often will epidermal glands and hairs, vascular bundles red to purple, other structures green. The flat prothallia and the protonema of ferns do well if fixed in Flemming's fluid and stained in Heidenhain's hematoxylin and orange G before mounting in balsam by infiltration, or they may be imbedded and sectioned for details of the sexual organs. The heterosporous Pteridophytes offer special difficulties in the hard walls of mature spores and spores containing gametophytes. As long as it is possible to cut at all, paraffin will probably be found the most satisfactory imbedding medium. Sections (as often in Pteridophytes) may not stick well to the slips unless a gum or gelatin affixing medium (p. 129) be employed.

XVII. Gymnosperms

The Gymnospermas like the Pteridophytes, are in most anatomical features readily adapted to the general methods of study, so that reference should be made to the first portion of the botanical chapter. A few special topics come up to be dealt with separately. These mainly relate to the handling of the megasporangial structures, the female gametophyte and the embryo.

In the Cycadales and Ginkgoales it is necessary, if sections are to show the general topography of the ovule (megasporangium, etc.,) that segments be cut from opposite sides to allow more ready penetration of the fixing fluids. These should be cut as deeply as possible, so that the cut surfaces are close to the particular structures developing at the selected stage (megaspore mother-cell, endosperm, embryo), but with a razor blade to avoid pressure on these structures, which in their early stages are readily

deformed. For more accurate fixation the upper portion of the endosperm or of the nucellus containing the stages especially desired should be cut out and fixed separately. The ovule will cut well enough in the paraffin until the stony layer becomes hardened. For the free nuclear stages especial care is required to keep from causing shrinkage, and a high percentage of osmic acid will be helpful in giving a thorough preservation of the cytoplasm. The pollen tubes with the sperm mother-cells (or the sperm) in their tips are best secured in this way, or (*Ginkgo*) by cutting away the lower part of the ovule and then slipping off the upper part of the nucellus, which comes away as a cap. At the apex of this the pollen tubes are well exposed for easy penetration of the fixing fluid, which should be a Flemming (p. 157). They may be imbedded, sectioned and stained with Heidenhain's hematoxylin, which shows the centrosomes and blepharoplasts well. Chamberlain* gives useful data on the times of maturation of the various stages. With *Pinus* it is necessary to dismember the cones for all but the very earliest stages in the first spring of their development. The individual scales soon become hard at the tips, and can advantageously be trimmed (after imbedding) back to the ovules. In old stages containing embryos the ovules may be isolated immediately and even trimmed on the side to facilitate fixation. Buchholz† has given directions for the isolation of the embryos of *Pinus* and other genera. Dissection from living material is best. The gametophytes are first isolated. Hold gently with light forceps by the broad end, and make a circumcissile cut about the narrow end with a needle shaped to an arrow-head tip and keenly sharpened. This end is gently removed, when by teasing into the end of the ovule the rosette ends of the suspensors are exposed and should be pushed out by the straightening suspensor shafts. By successive segmental cuts the distal portions of the suspensors, and eventually the embryos, are exposed, and finally the whole embryo complex removed. Dissection should be under a 0.3 gm. mol. sugar solution to prevent bursting of the embryo cells. Fixation in formol-alcohol is recommended, followed by staining in Delafield's hematoxylin or safranin. This is a proceeding for careful hands, the embryos being transferred with wide-tipped pipettes. If staining is accomplished in a small drop of stain, later to be flooded with water (or alcohol) before pipetting off, the embryos can be much more readily located. They are then dehydrated through glycerin and infiltrated in balsam or Venetian turpentine (p. 143).

XVIII. Angiosperms

1. Pollen investigations in connection with genetical studies are often desirable to determine the percentage of normal grains developed and the types of abnormal ones. Direct microscopical examination will indicate

* Chamberlain, C. J. Methods in Plant Histology. Chicago, 1924, p. 269.

† Buchholz, J. T. *Bot. Gaz.*, 1918, lxvi, 185.

badly shriveled grains, megacytes, etc. Germination tests will give additional information respecting pollen activity (below). Measurements of the length and growth rate of the pollen tubes in the styles (p. 180) will show the probability of the sperm being able to reach the embryo sacs. For more detailed information on grain sizes micrometer measurements may be made. For information on viability based on the stored food content the method presented by Blakeslee and Cartledge* may be tried:

"Flowers to be examined were collected in the morning, generally before the anthers had opened. The pollen was taken out of the anther with a needle and distributed in a drop of 45 per cent acetic acid slightly colored with iodine. This medium stains the contents of the grains but leaves the walls practically uncolored. Grains were recorded as abortive which were empty and shriveled. There is usually no difficulty in distinguishing bad from good grains. Counts were made of the two kinds of grains in a series of different fields of the microscope under low power. The abortive grains tend to collect at the edges of the drop. Their distribution was made more uniform by stirring with the needle and the counts were made more representative of the actual condition in the flower by examining, in succession, fields from one side of the preparation to the other."

In measurements care should be taken to make the observations soon after putting the pollen in the acetic solution, for even with the most cautious adjustment of the strength of the acid, swelling even to bursting, or shrinking, will result after thirty to sixty minutes. After familiarity with the material has been secured, differences in chromosomal number may be detectable in differences in the size of the pollen grains (courtesy of Cartledge). Chamberlain recommends for loose pollen of anemophilous plants that it be soaked for fifteen to twenty minutes before fixation in order that it may become turgid before fixing. Afterwards it may be fixed in bulk like minute organisms, washed by sedimentation, stained, dehydrated and infiltrated with balsam, or after dehydration imbedded and sectioned.

2. Pollen tubes are generally studied by culture methods.

Most pollens quickly lose their vitality, so germination should not be unnecessarily delayed. Some kinds grow well if simply spread on parchment paper or clean glass slips and maintained in a water-saturated damp chamber. Others require water, the drop being allowed to spread thin on a chemically clean slip so that oxygen may be more readily available. For most species success attends culture in a solution of cane sugar, the optimum strength of which varies with the plant over a wide range: *Papaver*, 1 per cent; Compositae 30 to 40 per cent. The sugar may be mixed with 1 to 2 per cent agar and the mixture allowed to stiffen on slips or in Petri dishes. After inoculation the slides must be kept in a damp chamber and the Petri dishes closely covered. The agar may be cut up into blocks and these sectioned after imbedding.

Pollen tubes in the pistil of a pollinated plant are best studied in position. The pistil may be cut off and fixed, and the tubes located in longi-

* Blakeslee and Cartledge. *Proc. Amer. Acad. Sci.*, 1926, xii.

tudinal paraffin or celloidin sections. The usual fixing fluids and imbedding methods may be adapted, but because of the general presence of air and spongy tissue in the style it is advisable to split it before fixing and to remove the air with an air pump. The common cytological staining methods may be employed for studies calling for such details, but for histological purposes a combination involving aniline blue is recommended, since this stains the callose of the tube wall especially readily from a dilute solution. A particularly valuable method has been perfected by Buchholz and Blakeslee* particularly for *Datura*, but widely adaptable. Their method may be quoted as follows (courtesy of J. L. Cartledge):

"The styles were . . . scalded in hot but not boiling water (about two minutes) their cortex slit lengthwise by passing them through a groove in which the sharp corner of a fragment of a razor blade protruded slightly. This treatment facilitated the removal of the cortical tissue by dissection, leaving only the central strand of conducting tissue with which the stigma is continuous at the end. These central cores were stained in Magenta (Acid Red), washed a little in water, and mounted whole on a slide, using concentrated lactic acid as a mounting medium and clearing agent. Balsam mounts were found not satisfactory but these lactic acid preparations have kept for more than six months. Pressure applied to the cover glass will spread this tissue out in a thin layer, and the pollen tubes may be seen even under low power (better after twelve to twenty-four hours) as dark red streaks embedded among the elongated pink stained cells of the conducting tissue. Germinated pollen grains are transparent and may be recognized only by their empty shells (the extine walls) while the ungerminated pollen will stain a deep red. This method makes possible reliable counts of the number of ungerminated pollen grains and the number of pollen tubes at any given time after pollination."

The use of lactic-glycerin (p. 111) instead of the simple lactic acid may be of some advantage as it is of slightly higher refractive index. The mounts may be sealed with the usual cements (p. 140).

* Blakeslee, A. F. *Science*, 1922, lv.

CHAPTER V

CYTOLOGICAL METHODS

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A. GENERAL CONSIDERATIONS

The development of cytological studies has reached such a stage that it has now become a matter of very great importance not only to have methods of the highest character, but also to make such a selection of material as will profitably repay study. Some consideration of the character of the material may therefore be worth while.

1. **Selection of material** should be discriminating. Sufficient studies have been made of the different plant and animal groups to indicate their cytological characteristics in a general way. Before undertaking an investigation, therefore, a review of the literature dealing with the different groups should be made in order to discover the best kind of material available for any particular purpose. While it is desirable to investigate any new types that present themselves, if a particular subject requires consideration it is wiser to select a form which has already demonstrated its worthiness. There are many elements that enter into such a decision, among which are the availability of the material at different seasons of the year, and convenience of handling in the laboratory. Sometimes, however, a form is exceedingly valuable, for instance, as genetical material and it then becomes necessary to discover, so far as possible, its cytological characteristics. Under such circumstances the best has to be made of poor material oftentimes, and the only recourse here then is to make the best possible use of our technical methods. When, however, a choice can be made, as has been indicated, the element of selection enters largely into the final results.

2. **Preliminary treatment of specimens** has much to do with the end result. If the material has not been fixed directly after being brought in from the field, specimens should be carefully housed in approximately normal living conditions. If we are dealing with animals, they should be kept in cages which are appropriate to their size and habits. In many cases it is of great importance how they are handled. For instance, it has been found in the case of the albino rat that if animals are gentled they exhibit very different reactions from those which have not been subjected to this preliminary training. While these differences are apparently largely physiological, they must have a structural basis. Physical conditions, such as food, light and temperature, also have marked influence upon the morpho-

logical characteristics of animals. Sometimes these influences are of a very profound sort. In the case of certain grasshoppers which were raised in the laboratory, if the animals were fed exclusively upon clover the cytoplasm of the germ cells became loaded with dense materials which were absent when the animals were fed upon a diet to which they were normally accustomed. In the absence of proper cleansing and light conditions, fungi may attack the animals, and, for the grasshoppers mentioned, it had much the same effect as followed feeding with clover.

3. **The condition of specimens at the time of fixation** requires consideration. The age of the specimen, of course, is directly related to the state of development of its cells, and an appropriate selection should be made in order to get the stages required. Only a knowledge of the rate of development will make this exactly possible, but in most cases, if studies of the germ cells are involved, an understanding of the breeding habits of an animal will indicate roughly the time at which the material should be put up. Even within the same group, however, there are marked differences in cellular history so that, for each species, it is necessary to acquire some familiarity with the type. Amongst the grasshoppers, for instance, there are certain ones, like the Truxalinae, where the adult animals show a quite satisfactory succession of developmental stages, whereas in the Acridinae the cells pass more rapidly through their development and show only later stages in the adult condition. In this latter case the cells give indications of senility and the conditions are not normal. Due care should therefore be exercised to see that the animals are in the proper stage to present cells of normal constitution. In the Hymenoptera, unlike many other insects, it often happens that all the cells of the gonad are in the same stage of development. Hance reports that by shaving off the hair of mice many dividing cells may later be found by sectioning the follicles.

Another matter which has to be considered is the metabolic condition of the specimen. It sometimes makes a great deal of difference whether the animal has been fed or starved. In general, of course, it should be in the healthy state which follows normal feeding, but in some studies conditions are revealed after starvation that are important for an understanding of normal cell functions. A feature of very great importance, which is often overlooked, is the length of the period between death of the animal and the preservation of its cells. As a rule, the material should be brought as rapidly as possible into the fixative, and it is often advantageous to apply the reagent to the cells in situ. As was indicated in Part 1 the method of killing also has an important bearing upon the structural conditions of the cells.

B. GENERAL METHODS

1. Fixation

A general consideration of this subject will be found in Part 1 and also in the chapters on Fixatives and Fixation, and Botanical Methods. In

cytological work, however, the choice and use of the fixative is of such significance that some special consideration may be warranted. In the first place it is necessary to decide whether the exact presentation of nuclear structures or of the cytosomic constituents is more important. While it is not true that there is such a marked difference in the particular reagents required as is commonly believed, yet there are certain practical considerations which operate in the choice. If one is inexperienced in the characteristics of the particular material which it is intended to study, the wisest procedure will be to select first one of the picro-formol-acetic combinations. The advantages of these, as is elsewhere indicated, are that they do not overfix and they are perhaps more generally adaptable than any other class of such fixatives. Their main disadvantages are that they do not preserve well all of the cytosomic elements, and besides certain nuclear aniline stains do not take so vigorously as after treatment with osmic acid mixtures. For these reasons it will generally be found advantageous to supplement the picro-formol-acetic fixations by others with chrom-osmic-acetic mixtures in which there are present varied proportions of acetic acid. These two classes of fixatives will be satisfactory in a very large proportion of the cases, even for final studies. After experience has been gained with a particular type of material it will be profitable many times for the investigator to experiment by varying the proportions of the ingredients which enter into the combinations. In extended studies of Orthopteran material and certain plants it has been found that these proportions have a marked influence upon the relative density of cell parts as well as upon their finer details. Also, as will appear in the fuller discussion under Fixatives and Fixation, and Botanical Methods, the presence of certain adjuvants like urea and sugars have pronounced effects when added to the fixatives. Since the value of all later steps depends upon the quality of the fixation, it becomes of first importance for the investigator to exhaust all of the possibilities of improvement at this stage before finally settling down to a study of the material. It cannot be too strongly emphasized that exact fixation is a primary requisite in all cytological work.

1. **Special Methods.** Special techniques have been developed in extensive studies of certain animal groups, some of which present unusual difficulties. Much of the work done upon mammalian cytology is valueless because the process of fixation has been improperly conducted. Within recent years two general methods of treating mammalian cells have been developed which produce excellent results. Already these have been given such thorough trial, under a great variety of conditions, that they may be said to have established themselves as the principal reliance in all studies where great exactness in the preservation of fine cytological details is required. These techniques involve not only a consideration of particular reagents, but also certain niceties in their use.

Flemming's fluid and Allen's B-15 have proved the most satisfactory fixing fluids for mammalian tissues, especially for chromosomes. In the United States the use of the former at near the freezing point has been found helpful. This method is described by Hance (p. 185). Allen's B-15 has been used by many workers and found very satisfactory.

a. Allen's Special Methods. Time of fixation in B-15 (for formula see p. 424) is usually one hour, if outer membranes have been removed and the fluid held at 38°C. This is based upon pieces of such tissue as rat testis and young rat brain not over 0.5 c.c. in volume. Longer fixation seems unnecessary and may be detrimental. After this time, some users have found that placing the tissue in Bouin's fluid is helpful. While the penetration of B-15 is better than that of Flemming's fluid, it is best to apply it to small pieces of tissue. Snip rat testis with the scissors so that many surfaces are exposed. Brains of very young rats do not much exceed 0.5 c.c. and they may be cut in half by sagittal division at time of fixation by using a thin safety razor blade, or they may be otherwise divided in order to let the fixative attack an unprotected surface.

If the tissue is held a few days in 70 per cent alcohol, with frequent changes, it is not only hardened but is freed from the remaining picric acid. The use of a few drops of a saturated solution of lithium carbonate at intervals hastens the removal of the picric acid. While some workers find the presence of picric acid not detrimental, my experience with mammalian tissue is that if much is present, infiltration with paraffin may be difficult and that thick sections (20 μ) do not adhere well to the glass. The higher grades of alcohol may be replaced by aniline with less shrinkage, as described on p. 190.

Other fixatives. B-20. I have recently found that a little 2 per cent osmic acid added to the B-15 mixture improves its action on mammalian chromosomes. As commonly made up, only half the chromic acid called for by the formula (p. 424) is used, and to each 50 c.c. is added 1 c.c. of Flemming's solution B. (1 gm. osmic acid in 50 c.c. of 1 per cent chromic acid. This is the equivalent of 2 per cent osmic acid.) For convenience it is named B-20. The after treatment is the same as for B-15. This fluid has been found very satisfactory also for eggs and spermatogenic tissue of the Cladoceran Moina macrocopa. Mix at time of using.

Double fixation, or the use of two fixing fluids, has been found useful in some cases. If much fat is present, Carnoy's fluid is first applied for from ten seconds to several minutes. This is followed by any other fixative. In Moina macrocopa eggs I have found that this treatment followed by B-15 eliminates the oil globules and fixes the remaining yolk substance and chromosomes very well.

General Remarks. Duration of Fixation. This varies greatly with different fixatives and tissues. Specimens remain indefinitely without injury in Bouin's fluid, but harden too much for good sectioning if left in B-15 over

two hours. Most people fix twenty-four hours in Flemming's fluid, but Professor H. de Winiwarter (Liège, Belgium) fixes from forty-eight to seventy-two hours. His formula is somewhat different from that in general use in this country. It follows:

(a) Osmic acid ..... 4.0 gm.
Chromic acid ..... 7.5 gm.
Distilled water ..... 950.0 c.c.

(b) At the time of using add 3 or 4 drops of glacial acetic acid to 20 c.c. of (a), or, as seems better, 6 or 8 drops of trichloracetic acid. He washes for twelve to twenty-four hours, and dehydrates carefully by graded steps, and clears in cedar wood oil (H. von Winiwarter, 1912). I have seen his preparations of human testis by this method and the chromosomes are beautifully distinct in all stages (Ezra Allen).

For a method of injection and methods of subsequent treatment see page 429.

b. Hance's Method for Mammalian and Avian Material. Flemming's strong solution to which has been added about one-half of one per cent urea, when chilled to the temperature of ice, gives an excellent preservation of the chromosomes in avian and mammalian cells. This mixture does not penetrate well, which necessitates the use of pieces of tissue preferably not larger than a small pea. In the case of chicken tumors, which are extremely dense, slices as thin as can be cut free-hand with a razor are dropped into the fixative. Chicken testes, which are also difficult to penetrate, should be thoroughly teased after being placed in the fluid. Avian and mammalian embryos have all their structures well preserved by this method without the necessity of teasing to aid penetration. Appropriate pieces of tissue are placed in the fixative. (For formula see p. 423).

Mix as needed, so that for every piece of tissue the size of a small pea from 10 to 15 c.c. of fluid is available. Place the bottles containing the fluid in cracked ice.

Fix four to twelve hours.

Wash in running water for twelve hours.

Dehydrate by the "drop method," displacing the water drop by drop with alcohol. While 70 per cent alcohol may be used for this purpose and the tissue stored in it I consider it more convenient to use 95 per cent alcohol and imbed the material at once. When the tissue has reached 95 per cent alcohol, change to fresh 95 per cent at least once, twice being better. Tissue remains in the last change of alcohol for from one to two hours.

Clearing. Cedar oil or oil of bergamot is used for clearing. It is preferable to add it to the alcohol drop by drop although satisfactory results are usually obtained by adding relatively large fractions to the alcohol which covers the tissue. Change the tissues to fresh oil two or three times. They may stay indefinitely in cedar oil although over night is usually a convenient interval for either oil. Before infiltrating with paraffin the oils may be either washed out with chloroform or placed in the paraffin without this step. When chloroform is used it is allowed to act from thirty to sixty minutes.

Imbedding Medium. Paraffin. The chloroform-covered tissues are warmed and melted paraffin is added several times during a two to four

hour period. The tissues are transferred to pure paraffin and allowed to remain for from two to four hours. The tissues may be handled in the same way when chloroform has been omitted, although the time allowed for infiltration must be longer as it takes more time to displace the heavier oils.

Section Thickness. Avian tissue gives best results when cut at 5 μ and mammals when cut at 10 μ.

Staining. The slides are bleached of the osmic acid stain in 70 per cent alcohol plus peroxide in proportion of about 4 parts of alcohol to 1 part commercial hydrogen peroxide. The slides remain in this solution twelve hours or longer. Iron alum hematoxylin has given the sharpest results for cytological studies (R. T. Hance).

c. Carothers' Method for Orthopteran Cells.

Picric acid, saturated aqueous solution	75 c.c.
Formalin, C. P.	15 c.c.
Glacial acetic acid	10 c.c.
Urea crystals	1 gm.

Warm slightly and stir thoroughly as the urea is added.

This solution is used if the material is to be kept in the fixation. It is satisfactory for work involving metaphase conditions.

For preservation of the finest cytological details add 4 drops of a 50 per cent aqueous solution of chromic acid to 5 c.c. of the above solution just before using. The tissue must then be removed from the fixative after ten to twenty-four hours, washed thoroughly in tap water (one or two hours) and run up to 70 per cent alcohol where it may be stored after most of the picric acid has been removed.

In case one wishes to use Flemming's triple stain, osmic acid must be added. Five drops of a 2 per cent solution to 5 c.c. of fixative is sufficient.

2. **Methods of Application.** It is sometimes a matter of great importance how the fixative is applied to the tissues. As is elsewhere indicated, there are two general methods, that of immersing the material directly in the fixative and that of injecting the fixative into the organ or whole animal to be studied. Sometimes in the case of smaller animals the whole organism is immersed in the reagent. It is not unimportant how the fixative is applied by immersion. For instance, in studies on Culex, some investigators reported the diploid number of chromosomes as 3 and others as 6. Upon careful investigation of the cause of these differences it was found that if the fat and trachea were not removed from the testis, the fixation was imperfect and the chromosome pairs ran together, reducing the number from 6 to 3. Although the organ here is very small, and one would naturally think that fixation might be easily accomplished, the neglect to remove the fat and trachea was responsible for such an important difference as this. It should be the rule, therefore, so to manage the process that the fixative is brought into as direct and immediate contact with the tissue as is possi-

ble. A further discussion of this topic will be found in the chapter on Fixatives and Fixation.

In the case of large organs it is important to effect this intimate contact between the reagent and the tissue by injection through the circulatory apparatus. The difference in the effect produced by fixation of mammalian testes through immersion and by injection is very marked. Ordinarily, in the former case, the histological details are very poorly preserved, there being extensive shrinkage of the germinal elements from the connective tissue about them. In a preparation well preserved by injection, on the contrary, the shrinkage is entirely absent and the cells are everywhere in intimate contact. In studies upon large organs, therefore, it should always be the endeavor to try out the process of fixation by injection. It is described in detail by Allen in the chapter on Fixatives and Fixation.

3. **Physical Conditions during Fixation.** The concentration of the fixative, the proportion of its parts, the presence of adjuvants and the degree of temperature, are all elements that require extreme care in cytological technique. These are discussed at length in Part 1 and in the chapters on Fixatives and Fixation, and Botanical Methods. It is perhaps sufficient to call attention to these points here and to emphasize their great importance.

4. **Period of Fixation.** This is determined by the size of the specimen and by its character. In general it is desirable not to prolong the action beyond the time necessary to secure accurate fixation. This varies also with regard to the character of the reagent. It is quite impossible to give any general rule which can safely be applied to all types of material and to the reaction of all fixatives, but in general it may be said that a piece of ordinary tissue not exceeding 5 mm. in diameter may be fixed within two to twelve hours with safety in most fixatives. In special cases, on the contrary, it is often desirable to prolong the period of fixation to great lengths in order to secure specific effects. This is particularly true in the case of mitochondrial studies and in certain neurological practices. It is perhaps a safe general plan to start with a minimum period and extend this only as experience indicates that it is desirable.

II. Washing

A description of this process will be found in Part 1 and also in the chapter on Fixatives and Fixation. It will suffice here to indicate that in cellular studies extreme care is necessary, since, after chrom-osmic-acetic fixations, for instance, either a mitochondrial stain or a chromatic stain may be secured with hematoxylin, depending upon the extent of washing. Puzzling artifacts may result from insufficient washing, especially after the use of mercuric chloride. In general, the quality of staining is much influenced by the degree of washing of the fixative.

III. Dehydrating

Even the best fixation may be spoiled by after treatment and this is particularly true during dehydration. The absolute rule here is that the transfer of the material from aqueous solutions to alcoholic must be made in the most gradual manner. To accomplish this the tissues may be run up through a finely graded series of alcohols allowing sufficient time in each for a complete replacement of the lower percentage of alcohol, or, preferably, the alcohol may be added drop by drop to the water in which the specimen is placed until the concentration of alcohol is high. A description of this method is given by Allen as follows:

> In this process certain desiderata need to be kept in mind. The first is, to make the process as short as possible, especially in the latter part, as at this stage the shrinkage is great. Tissues fixed in Bouin's fluid or B-15 seem in better condition if held two or three days in 70 per cent alcohol. Flemming's fluid and most fluids which contain corrosive sublimate harden as well as fix.

The following method has proved very satisfactory for maintaining the normal relationships between tissues of considerable difference in texture, as the interstitial cells and the tubules in rat testis, and mesenchyme and ectoderm in mammalian embryos. (Allen, 1919.)

The new fluid is added one drop at a time, while the fluid containing the tissue is agitated by either mechanical stirring or by a current of air bubbling through it. The agitation insures that the surface of the tissue is constantly bathed in a fresh fluid. Bubbling air may be introduced from any source of compressed air. The accompanying cut (Fig. 2) shows an adaptation of the pressure bottle for this purpose (P.B.). One may substitute an atomizer bulb connected to the pressure bottle for the water bottle (W.B.) as shown in Figure 1. Sufficient pressure to last for some time may thus be obtained. A slight current in the fluid is sufficient to distribute rapidly the new drop of fluid as it enters the container (C). An automatic siphon (S) carries away the excess into a waste jar (Waste). The sulphuric acid drying bottle may be connected in the air tube when adding the clearing agent.

The following brief outline of procedure is based upon the treatment of a piece of tissue about 0.5 c.c. in volume which has been fixed in B-15. Agitation is understood to be maintained throughout the process, even if no fluid is being added. The new fluid is added at the rate of one drop per second unless otherwise stated. The left hand column indicates the fluid in which the tissue is at that stage. Remove the tissue from the fixing fluid, rinse in 5 per cent alcohol, and place in 5 per cent alcohol.

1. 5 per cent alcohol. Drop in 10 per cent alcohol plus a few drops of saturated solution of lithium carbonate, until tissue is in practically 10 per cent alcohol.
2. 10 per cent alcohol plus lithium carbonate. Let remain two hours with agitation.
3. Fresh 10 per cent alcohol. Drop in 50 per cent alcohol plus about 1 per cent lithium carbonate until tissue is in approximately 30 per cent alcohol.
4. 30 per cent alcohol. Drop in 50 per cent alcohol plus an equal part of aniline, one drop about every five seconds, until the tissue is in approximately the new mixture.

5. 50 per cent alcohol and aniline, equal parts. Drop in equal parts of 70 per cent alcohol and aniline, one drop in five seconds.

6. 70 per cent alcohol and aniline, equal parts. Drop in pure aniline still more slowly—

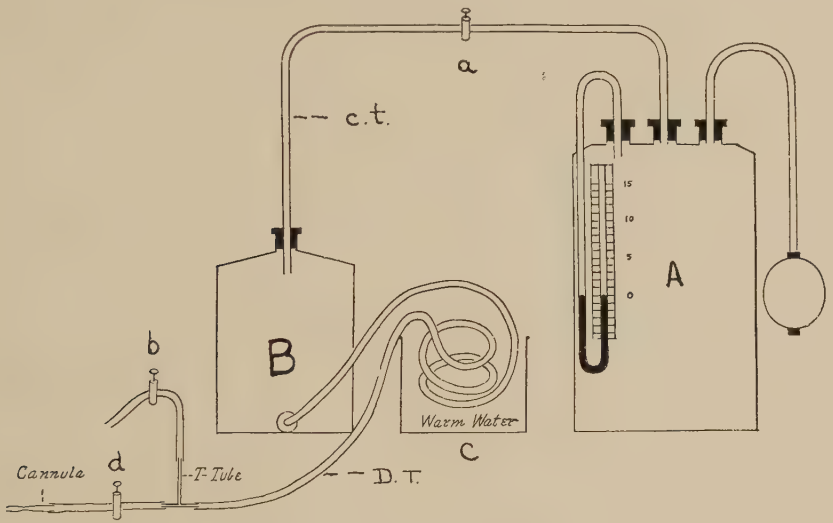

FIG. 1. Apparatus for injecting.

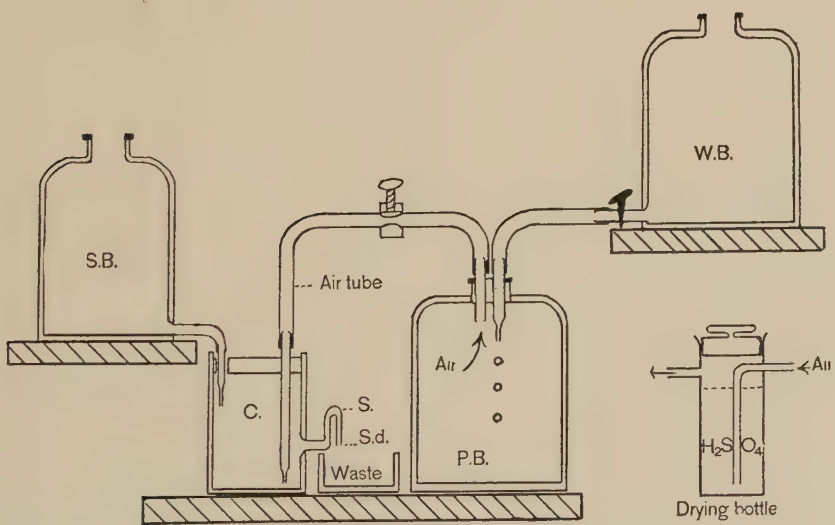

FIG. 2. Agitator.

one drop in ten seconds. Continue until tissue is clear throughout, when dehydration is complete, and clearing may be begun by the same method.

If a whitish precipitate appears at any step after aniline is begun, it indicates that not all of the fixative has been removed, as aniline and the fixative are incompatible. In such cases the tissue must be returned to the former fluid for a longer time. If alcohol instead

of aniline is employed, as it may be for some relatively hard tissues, removal of all the fixative is not necessary. (See remark on this subject on page 184.) Do not use aniline after Flemming's fluid. The tissue turns very dark and seems impossible to bleach.

Dehydration may be carried on by the more laborious method of adding the new fluids by 5 per cent or in some cases 10 per cent steps without agitation, but it is time-consuming.

If large numbers of pieces of tissue are to be done at once, the apparatus described on p. 433 may be used.

IV. Clearing

The process is an intermediate step between dehydration and infiltration in the paraffin method, and grades by varying degrees, according to the reagents used, into these other steps.

Dehydration is commonly completed in alcohol, but in the case of delicate tissues for cytological work it is best to avoid the higher grades of alcohol and to utilize some such agent as aniline oil in order to escape their stronger action. The processes, therefore, cannot be regarded alone, but must be considered in relation to dehydration and infiltration. If dehydration has been completed in 95 per cent alcohol or absolute, there are available quite a number of clearing agents which may be utilized to displace the alcohol. For many cytological purposes the benzine ring series, benzol, toluol, and xylol are particularly appropriate preceding the paraffin bath. For mammalian testes Allen has found that methyl-salicylate gives unusually good results, while for brain tissues he prefers bergamot oil.

Following fixation in B-15 his experience has been that aniline is most desirable and refers to the method as follows:

"For most purposes alcohol is an adequate dehydrating agent. However, for mammalian chromosomes fixed in B-15 the use of aniline has proved very helpful (Allen, 1916; Painter, 1924), as it produces less shrinkage. The purest aniline obtainable is usually somewhat reddish, but by distillation it becomes nearly colorless, and does not stain the tissues. Aniline is perfectly miscible in 50 per cent alcohol equal parts. This mixture passes readily into 30 per cent alcohol. Hence pure alcohol higher than 30 per cent may be avoided." (For details of method see p. 188.)

If aniline is used to complete dehydration and for clearing it is best to remove this with some agent like chloroform of higher volatility and greater miscibility with the paraffin.

For very delicate cytological work the same precautions that apply to dehydration also hold for clearing. It is necessary to avoid sudden changes in concentration of fluids and to minimize distorting effects of diffusion currents set up between different substances. Accordingly there must be provided a gradual passage through a series of mixtures of different relative concentrations, or the addition of the clearing fluid drop by drop to the dehydrating agent in which the specimen is placed. Allen's description of the drop method of dehydration therefore can be applied to clearing.

Whatever the agent employed or the method of its application, care should be exercised not to expose the tissue longer than necessary to the

action of the clearing medium. Practically all such substances have a shrinking and hardening effect which should be minimized by the briefest possible action. If the essential oils or aniline are used the completion of the process can be determined by the translucent appearance of the mass of tissue (see Part 1). Careful experimentation will reveal for any particular tissue the clearing medium which will produce the least shrinkage and in this manner it can almost be eliminated. Thus cinnamic aldehyde will clear delicate chick embryos with practically no reduction in size whereas xylol will reduce them to three-fourths their original bulk. When delicate cellular detail is involved no pains should be spared to preserve this against distortion by all reagents employed.

V. Infiltrating

It is a general practice amongst botanical cytologists to make a very gradual substitution of the clearing agent by paraffin. (See the chapter on Botanical Methods.) Such a procedure is less common amongst animal cytologists, but in many cases it is doubtless true that a better result would be obtained than by the method of transferring the material directly from the clearing fluid into the melted paraffin. In case of many animal tissues, however, it is quite satisfactory to make such a sudden transfer and thereby the hardening action of the clearing agent is somewhat minimized. When new material is being investigated it would be a good practice to try out the two methods and follow the one which gives the better results. The danger involved in the use of paraffin results from its hardening action when melted. Some workers, therefore, prefer to infiltrate first with a paraffin of low melting point, following this by final immersion in the higher grade desired for sectioning. The endeavor should always be to reduce the exposure of the tissue to the melted paraffin to the lowest limits and also to use the softest paraffin that will produce good sections. The latter alternative, however, has very limited range. Since higher temperatures produce the most disturbance in the tissue, the temperature should be kept as near that of the melting point of the paraffin as possible. There are numerous mechanical devices for securing this condition, but the simple overhead application of heat described in Part 1 gives a most valuable and flexible means for securing this result.

What has just been said with regard to the harmful effects of melted paraffin at high temperatures is undoubtedly true in most cases, but Foot and Strobell* report that the eggs of Allolobophora are not injured by the use of paraffin melting at 74°C. Quite in contrast to usual practice also, in order to get very thin sections, they cut at a temperature of 25°F. Like all other rules on technical matters the ones relating to handling paraffin must be general, and entirely subject to modification according to experience.

* Foot, K., and Strobell, E. C. *Biol. Bull.*, 1905, ix, 5.

In his work on mammalian cells Allen finds that the process of infiltration by degrees is as important as any other step. It should be at as low a melting point as possible for sectioning. A combination of ordinary parowax with about 4 per cent bayberry wax is recommended as an excellent medium. Pure parowax is used by many workers instead of that prepared for a certain melting point. Rubber paraffin (p. 476) with a low melting point is probably the best, as it is very flexible at any temperature or thickness of ribbon. Small blocks may be cooled on ice and sectioned at 5 μ on a warm summer day, when ordinary paraffin would be too soft. If possible, keep below a temperature of 52°C.

The paraffin may be first added to the clearing agent in small pieces, and these allowed to dissolve at room temperature. Chloroform has the advantage of absorbing a large amount of paraffin at this temperature. Some workers saturate it before applying heat. Keep tissues in the melted paraffin only long enough to insure complete infiltration. Shrinkage is likely to be great in any case.

If collodion is used instead of paraffin, less shrinkage results than with paraffin at its best. Follow either 100 per cent alcohol or aniline with a mixture of equal parts of 100 per cent alcohol and ether, and this fluid with 2 per cent collodion. Use plenty of this and let it slowly evaporate to a stiffness suitable for sectioning. The evaporation should take from ten days to two or more weeks.

VI. Sectioning

Sections which are quite satisfactory for ordinary histological work may be entirely unsuited to use for finer cytological studies. Unless the conditions are of the very best with regard to the character of the material, the relation between the material and the imbedding medium and the perfection of the knife edge, the sections will be faulty. Especially in chromosome studies, on material where the formed elements may readily be dislodged by an imperfect knife edge, it is necessary to use every precaution to avoid injury to the cells. These conditions become especially difficult of fulfillment when extremely thin sections are desired. Aside from having the microtome in perfect mechanical condition, and the knife edge as keen and smooth as possible, the best aid in getting exact sections is to have the melting point of the paraffin accurately adapted to the temperature of the room when the sections are cut. Finally, the inclination of the knife to the plane of sectioning should be very carefully adjusted to reduce the amount of compression to the minimum. In case very thin sections are desired the water method of Huber is extremely useful. It has been used by Heuser and is described by him elsewhere (p. 478).

The thickness of sections is dictated largely by the size of the cells present and by the character of the study to be made. Precision of staining is reduced if sections are thicker than the diameter of nuclei since differen-

tiation is hindered by their uncut membranes. Some sections should therefore be cut thin so as to expose nuclei to the direct action of stains. If very exact studies of delicate details are contemplated, very thin sections (1 μ to 5 μ) should be made. On the other hand, if general relations are to be studied in the cell, or if counts of entire chromosome complexes are to be made, thick sections are preferable. It is a safe rule, especially in the use of unfamiliar material, to cut thin, medium and thick sections from each specimen. An inspection of readily prepared smears will indicate approximately the required thickness of sections.

VII. Spreading

Much of the earlier cytological work was accomplished on sections which were affixed to the glass slip by pressing them down upon a thin film of Mayer's albumen. Since considerable compression occurs at the time of sectioning, especially with thin sections, much distortion of cell elements was produced. In all exact studies where the relations of cell parts are important, it is necessary to extend the sections to the original dimension of the block from which they were cut. To do this the common practice is to apply heat to the sections while they are floated upon either distilled water or a very dilute solution of albumen. By this means all irregularities are removed and the original proportions of the cell parts are restored. If the objects studied are eggs the sections should show them with circular outlines rather than oval ones. Such a test can only occasionally be applied, of course, but some means for determining the complete extension of a section is desirable. This is fairly approximated when the individual sections show the same dimensions as the surface of the block from which they were cut. This precaution is especially necessary if comparative measurements of cell elements are contemplated, for not only is a differential compression avoided, but also the errors that are introduced by foreshortening of structures lying in folds of sections. Spreading is an operation of seeming unimportance, but upon the care with which it is carried out depends much of the value of the preparation. Here, as in all other steps of preparation, only the best attainable results should be tolerated. A good slide may serve many unforeseen purposes, whereas one which may be technically sufficient for an immediate use may be so imperfect as to be otherwise valueless.

VIII. Staining

There are certain stains which are of particular importance in cytological work and constitute a standard resource in such studies. In the first rank of these stand the iron hematoxylin method of Heidenhain and the tricolor method of Flemming. So far as nuclear elements are concerned these two combinations suffice for almost all types of cytological work. Since a description of stains and staining will be taken up in a separate

chapter, no further reference to the operation of this part of the process will be given here. (See Stains and Staining.) In most material it will be found that nuclear structures particularly, in various stages of development, stain with different intensities so that in one slide it is difficult to find optimum conditions for all. Because of this and for other reasons it is of advantage to have slides some of which are lightly stained, while others are darker. *A fortiori* the differences apparent in hematoxylin and tricolor stains of the same material make the practice of using both desirable as a matter of routine. The transparency of aniline preparations and the contrasts in color they show in the same elements at different stages make them very valuable. With the improvements in the optical performance of modern microscopes the use of artificial light has become almost universal with the high powers used in cytological work. Colors which contrast well with daylight illumination often fail entirely with artificial light, so that it becomes necessary to test out the dyes which enter into combinations under the optical conditions used. For instance, the safranin formerly recommended in the tricolor stain is much too purple to contrast well with the gentian violet, and some more yellow variety is therefore required. By the choice of appropriate light filters somewhat the same effect may be secured if necessary.

Special stains are required for cytosomic constituents and these are described in the section of this chapter devoted to such structures.

IX. Collodion Sections

In cytological work the paraffin method is used almost exclusively. This practice is largely justified by its convenience and general accuracy, but there are certain advantages in the use of collodion which would practically require that at least some of the material used in each investigation should be prepared according to this method. While it is difficult to cut thin sections in collodion, by proper manipulation this may be readily accomplished, and, by using the appropriate means, serial sections can be readily mounted. Some of the advantages in the use of the collodion method appear prominently when studies of prophase figures in the germ cells are involved. There is less danger of shrinkage than with paraffin and minute separations of parts often appear with greater precision and permanence than they do in paraffin sections. It is true, also, that after some fixatives which give very poor results when sectioned by the paraffin method, collodion sections may present accurate and brilliant pictures of normal conditions. For instance the chromosomes of Orthopteran spermatocytes after fixation in Helly's fluid showed extensive shrinkage and it was quite impossible to use them, whereas in collodion sections they were of very excellent quality.

While the paraffin method may be applied almost universally there are certain resistant objects which have so far defied sectioning in paraffin.

Among these are Orthopteran eggs. For such objects the collodion method offers a welcome alternative, and, as thus applied, is described as follows by Josephine W. McNabb:

The preparation of the freshly laid grasshopper egg is difficult, due to the large amount of yolk and thick chitinous chorion present. Carnoy-Lebrun penetrates the chorion and yolk, giving a thorough and good chromosome fixation. The separated eggs are treated about ten minutes, allowing one cubic centimeter of fixing fluid per egg. After about five minutes the chorion is punctured with a fine steel needle. (The puncture should be made in a lateral position, thus avoiding the critical region about the caudal end. The yolk should be sufficiently hardened so that it does not exude from the puncture.) From the fixing fluid they are transferred immediately to weakly iodized 70 per cent alcohol for about twenty-four hours, or until the solution is no longer decolorized. The eggs are then removed to 70 per cent alcohol where they may be left indefinitely.

The collodion method is the only thoroughly successful one of sectioning. Paraffin is not satisfactory on account of the difficulty in cutting the brittle yolk, and because heat causes clumping of the chromosomes and shrinkage of the large cells. The ordinary means of dehydrating and of infiltrating with collodion may be followed. It is necessary to use thick collodion and allow the infiltration to continue for two to four weeks. The chorion should be punctured in a lateral region under the collodion.

Longitudinal or oblique sections are more conveniently prepared than cross sections. In making serial sections the eggs are cut at 12 micra, preferably with a Minot precision microtome. As the sections are cut each one is pulled to the back of the knife with a sable brush. Here they are arranged serially in parallel rows. It is necessary to keep the sections and the blocked egg constantly wet with 60 per cent alcohol. When the entire egg is cut the sections are transferred in order to a chemically clean slip. This is accomplished by first pulling them onto a safety razor blade with a dissecting needle, and then onto the slip. The length of the rows should be made to correspond to the length of the coverslip used, and the same number of sections should be in each row.

After each slip is filled (an egg requires approximately two slips) the moist sections are blotted with filter paper to press out all excess alcohol. A thin solution of collodion is quickly flooded over the sections with a pipette, and this coating is immediately rinsed with a mixture of equal parts absolute alcohol and ether to remove as much of the collodion as possible, but still leave sufficient to prevent the sections from becoming displaced. The slip on which the sections are mounted is then placed in 70 per cent alcohol until it is convenient to stain. The sectioned egg, thus mounted, is mordanted in 4 per cent iron alum, stained in a ½ per cent solution of Heidenhain's hematoxylin and mounted in damar. This method may be applied to other large insect eggs as well and the transfer of sections

in serial order by the use of tissue paper (Part 1, p. 28) may be substituted for the plan given.

The method of preparing collodion sections as ordinarily practiced is described in Part 1 and in most cases requires no particular modification aside from that involved in securing very thin sections.

X. Smear Methods

Of particular value in cytological investigations, especially in regard to germ cells, are the so-called smear methods. The general effect of the use of any of these is to spread out the cells in a thin layer on the glass slip or cover. By varying the pressure there are produced regions in which cells present distinct degrees of compression, extending even to the bursting of the cell and the liberation of its elements. Except in the latter case, the entire cell is preserved so that certain inaccuracies, which may be noticed in the study of serial sections passing through an individual cell, are avoided. Smear methods, therefore, are particularly valuable in the study of chromosome complexes where numerical relations are of primary importance. As was indicated in Part 1, it is not wise to rely entirely upon the appearances found in smear preparations and they should always be carefully checked up with similar conditions in sections. Judiciously employed, smear methods are of the very greatest value and are always to be resorted to when practicable. Until within recent years this method has found little application in the study of plant cells. Recently, however, Taylor and Kaufmann, by a simple modification in the method of applying pressure, have been able to achieve valuable results in the study of both somatic and germ cells of plants. The very great value of the method in laying bare details of cell structure, by separating the parts through pressure, is demonstrated. For an account of this application see Chapter IV.

While the smear method is simple in principle, there are difficulties in its application which make it desirable to give somewhat in detail the steps involved. Accordingly the application of the method to the testis of an insect will be described.

First, the organ is exposed by a ventral incision of the body and it is then freed from the surrounding tissues. Previously, appropriate sized cover glasses have been cleaned and are ready for use. In selecting these covers they should be of such a size as to supply space for the entire contents of the testis, or, if this be too large, for an appropriate sized piece of the organ. This is an essential step since if the material is too great some will be lost, whereas if it is too small the pressure and traction may be excessive and destroy many of the cells. The best conditions are provided when the material just fills the space between the covers.

With the material thus placed a slight pressure is applied by means of forceps. This is a delicate portion of the operation and should be judged by the extent to which the follicles are ruptured, permitting the germ cells to separate thinly between the cover glasses. If the testis is from a young animal, the connective tissue is not very resistant and spreading is easily accomplished, whereas if it is from an adult with a greater amount and density of connective tissue the pressure will have to be increased correspondingly.

There is also a considerable difference between species in this regard and only experience with a particular material will permit one to operate successfully in every case.

Assuming that the cells have been properly spread, the next operation is to slide the covers apart in the plane of their contact with a fairly rapid and uniform movement. If there is a variation here it will be reflected in the irregularity of the film. The object is to secure a uniform distribution of the cells in a single layer. It is sometimes desirable to proceed one step further in order to rupture some cells, thus liberating their contents. Especially in the study of the minute details of chromosome structures, such free elements are of particular value.

As soon as the two covers are separated they should at once be inverted upon the fixing fluid so that the film is directly exposed to its action. It is absolutely necessary in most cases to avoid any drying of the cells. Fixation of films may be accomplished by almost any of the common reagents, but, as in other instances, the picro-formol-acetic mixtures and Flemming's fluid commonly give the best results. Since the film is thin, fixation occurs very rapidly and an exposure of a few minutes is sufficient. After fixation the film is treated very much as though it were a section and the processes of staining and mounting are carried out according to directions for sections. For staining it is often of value to have one of the pair of smears stained in iron hematoxylin and the other in Flemming's tricolor when the fixation permits. If large numbers of cover glasses are handled at one time they may be carried through together in racks made for the purpose.

A variation of this process which was introduced by Foot and Strobel for handling Hemipteran male germ cells is carried out as follows:

The testis is seized by a pair of forceps and dragged back and forth in a regular pattern on the surface of a clean glass slip until the entire contents are distributed in a film. This is merely allowed to dry in order to secure fixation and is then stained with Bismarck brown and mounted in balsam. Such a procedure is quite inapplicable to Orthopteran germ cells and to those of many other insects, but works well with the Hemiptera. Still another variation of the smear method was introduced by Foot and Strobell* for the study of the eggs of Allolobophora. In the operation of this method the individual eggs are isolated in small drops of water on a clean glass slip and each of them is pricked with a needle and the contents allowed to flow out and distribute themselves in the small quantity of water present. By simple evaporation of these small droplets the material is fixed and is then ready for staining and mounting. Both of the methods of Foot and Strobell are somewhat unusual and are particularly adapted to the materials which they employed. The results obtained, however, are so good that they warrant a trial in other cases.

XI. The Aceto-carmine Method

A method very valuable for rapid results is one employing Schneider's aceto-carmine, by means of which the cells are fixed and stained simultaneously. This was utilized to great advantage by Miss Stevens in an extensive series of studies on germ cells of insects. It has the disadvantage of causing extensive swelling of the chromosomes accompanied by much internal distortion of detail and the results lack permanence. If it is desired to preserve the specimens for a limited time this may be accomplished by ringing a cover glass with some cement which will prevent evaporation. In recent years Belling has somewhat modified this method and has made

* Foot, K., and Strobell, E. C. *Am. J. Anat.*, 1905, iv, No. 2.

much use of it in his studies on plant cells. For further discussion of this application of the method see Chapter IV.

C. SPECIAL METHODS*

I. The Mitochondria

1. Examination of Living Cells Unstained. Mitochondria may be studied in living cells teased out in serum or physiological salt solution without the addition of any stain by direct illumination or with the dark field. Their appearance by the latter method is beautifully illustrated by Strangeways and Canti (1927).† In animals they are perhaps best seen in the acinous cells of the pancreas where they are of unusually large size and may be recognized by their filamentous shape. Favorable plant material is afforded by pumpkin hairs, for the examination of which during life Maximow's (1913)‡ paper will serve as a guide. Mitochondria may also be readily studied by direct and oblique illumination in the living and growing cells of tissue cultures.

2. Supravital Staining. The most satisfactory dyes are Janus green B, Janus blue, Janus black I, and diethylsafranin. The first three are the only known specific stains for mitochondria. Their chemistry is described by Cowdry (1918). They may be applied by immersion or injection. Janus green B is diethylsafranin-azo-dimethyl-aniline chloride.

a. Immersion. The best results are obtained with blood (Cowdry, 1914) as follows:

Janus green B should be employed in a concentration of about 1: 10,000 in 0.85 per cent sodium-chloride solution. A drop should be placed on each of a series of six or more slips. A small amount of freshly drawn blood is then added to the dye and a cover glass is immediately dropped on it. No attempt should be made to mix the blood with the stain before covering.

The preparations should now be examined. Almost immediately one of them will begin to show mitochondria, first in the lymphocytes and later in the granular leucocytes. Soon the mitochondria will be stained in all of them. Under favorable conditions the color will last for several hours. Evaporation may be reduced by putting a ring of vaseline around the edges of the cover glass.

It is difficult in this way to secure a good coloration of mitochondria in nerve cells and most plant cells because the dye penetrates poorly.

b. Injection. This method is most satisfactory with the pancreas (Bensley, 1911) and salivary glands, but may be employed with all organs possessed of a rich blood supply. It does not, however, work well with the brain.

The animal is killed and Janus green B is injected into the left ventricle or aorta in a concentration of 1: 10,000 of salt solution by gravity pressure. In order to obtain a good

* Section on Special Methods by E. V. Cowdry.

† Strangeways, T. S. P. and Canti, R. G. *Quart. J. Micr. Sci.*, 1927, lxxi, 1.

‡ Maximow, A. *Anat. Anz.*, 1913, xliii, 241.

penetration the return flow through the inferior or superior vena cava, as the case may be, should be momentarily cut off by artery clamps. After about 10 minutes' perfusion, small portions of the gland may be removed and examined for mitrochondria. When the desired intensity of staining has been reached, the entire gland should be placed in salt solution pending examination.

3. Fixation and Staining.

a. Altmann's (1890) aniline-fuchsin-picric acid method (slightly modified):

(1) Fix small fragments, not more than 2 mm. thick, in 5 per cent potassium bichromate 10 c.c. and 2 per cent osmic acid 10 c.c., twenty-four hours.

(2) Wash in water one hour.

(3) Dehydrate in 50, 70, 95 per cent, and absolute alcohol twelve to twenty-four hours each.

(4) Half absolute alcohol and xylol, three hours.

(5) Xylol, three hours.

(6) 60°c. paraffin three hours. Imbed. Cut sections 3 μ to 4 μ, and fix to slips by albumen-water method.

(7) Pass down through toluol, alcohol, 95, 70, and 50 per cent alcohol, about thirty seconds each, to aq. dest. in staining jars.

(8) Stain for six minutes in Altmann's aniline fuchsin (aniline water 100 c.c., acid fuchsin 20 gm.). The stain may be poured onto the slide and the whole gently heated over a spirit lamp.

(9) Blot and differentiate by carefully flooding the section with a mixture of 1 part of sat. alc. solution of picric acid and 2 parts of aq. dest., added with a pipette. During this operation the color can be best seen against a white background.

(10) Rinse rapidly in 95 per cent alcohol. Pass through several changes of absolute into xylol and mount in balsam.

In this way the mitochondria are stained a beautiful crimson color against a bright yellow cytoplasm. It is the oldest and in many respects the best of mitochondrial methods, but it has two disadvantages—the fixative penetrates badly and the colors fade rapidly. Accordingly, neutral balsam or cedar oil adapted for immersion objectives should be used, the specimens should not be exposed to direct sunlight or to heat, and they should be kept in a dry place.

Bensley proceeds as follows:

(1) 2.5 per cent potassium bichromate, 8 c.c., 2 per cent osmic acid 2 c.c. glacial acetic acid 1 drop, twenty-four hours.

(2) Wash, dehydrate, clear, and imbed (p. 10), except that bergamot oil is substituted for xylol.

(3) Pass section down to water.

(4) Dip in 1 per cent potassium permanganate about one minute.

(5) Rinse in 5 per cent oxalic acid same time and wash in water.

(6) Stain with aniline fuchsin as indicated.

(7) Differentiate in a 1 per cent aqueous solution of methyl green.

(8) Rinse rapidly in 95 per cent alcohol. Pass through several changes of absolute into xylol and mount in balsam.

The use of permanganate and oxalic acid corrects excessive mordanting with the osmic acid and bichromate. It may sometimes be dispensed with. The methyl green, which was first used in this way by Galeotti, is a much finer contrast stain than the picric acid and is also more permanent. The precautions already mentioned against fading should be observed.

A second modification (Cowdry, 1918) may be given:

(1) Regaud's mixture (3 per cent potassium bichromate 20 c.c. and formalin 5 c.c.). The commercial formalin may profitably be neutralized by saturation with magnesium carbonate. The mixture may be applied by immersion or injection, the latter being recommended for large objects. It should be changed every day for four days and be kept in an ice-box (though this is not essential). Mordant for eight days in 3 per cent potassium bichromate, changing every second day.

(2) Wash in running water over night.

(3) Dehydrate, clear, and imbed as indicated (p. 10).

(4) Pass slides to water as indicated.

(5) 1 per cent potassium permanganate thirty seconds, but time must be determined experimentally.

(6) 5 per cent oxalic acid thirty seconds. Steps (5) and (6) may usually be dispensed with.

(7) Rinse in several changes of distilled water about one minute. Incomplete washing prevents staining with fuchsin.

(8) Stain in Altmann's aniline fuchsin made up as follows: Make a saturated solution of aniline oil in distilled water by shaking the two together. Filter and add 10 gm. of acid fuchsin (Duesberg) to 100 c.c. of the filtrate. The stain should be ready to use in about twenty-four hours. It goes bad in about a month. To stain, dry the slide with a towel, except the small area to which the sections are attached; cover the sections with the stain and heat over a spirit lamp until fumes, smelling strongly of aniline oil, come off; allow to cool; let the stain remain on the sections about six minutes; return the stain to the bottle.

(9) Dry off most of the stain with a towel and rinse in distilled water, so that the only remaining stain is in the sections. If a large amount of the stain is left it will form a troublesome precipitate with methyl green; on the other hand, if too much stain is removed the coloration of the mitochondria will be faint.

(10) Allow a little 1 per cent methyl green, added with a pipette, to flow over the sections, holding the slide over a piece of white paper, so that the colors may be seen. Apply the methyl green for about five seconds at first and modify as required. This is the crucial point of the method.

(11) Drain off excess of stain, plunge into 95 per cent alcohol for a second or two. Then rinse in absolute alcohol, clear in toluol, and mount in balsam.

(*a*) The methyl green may remove all the fuchsin, even when applied only for a short time. This is due to incomplete mordanting of the mitochondria by the chrome salts in the fixative. It may be avoided by omitting steps (5) and (6), or by treating the sections with 2 per cent potassium bichromate for a few seconds just before staining (as advised by Bensley). The action of the permanganate and oxalic is to remove the bichromate.

(*b*) The fuchsin may stain so intensely that the methyl green removes it imperfectly or not at all. This, on the contrary, is due to too much mordanting. It may be corrected by prolonging steps (5) and (6).

(c) Sometimes, after obtaining a good differentiation, the methyl green is washed out before the slide is placed in toluol, in which event omit the 95 per cent and pass to absolute direct.

This fixative is a good penetrator, in which respect it is much superior to Altmann's fluid or Bensley's mixture. The staining is satisfactory and uniform. Excellent results are obtained with plants as well as with animals. It is recommended for pathological examinations.

b. Benda's (1901) crystal violet alizarin method:

(1) Flemming's fluid, eight days.

(2) Wash one hour, half pyroligneous acid and 1 per cent chromic acid, twenty-four hours.

(3) Two per cent potassium bichromate, twenty-four hours.

(4) Wash in running water twenty-four hours, dehydrate, and imbed in paraffin.

(5) Mordant sections in 4 per cent iron alum, twenty-four hours.

(6) Rinse in water and bring into an amber-colored solution of sodium sulphalizarinate, made by adding a saturated alcoholic solution to water, twenty-four hours.

(7) Blot with filter-paper and stain in equal parts of crystal violet solution and water. The crystal violet solution made of sat. sol. crystal violet in 70 per cent alcohol 1 volume, alcohol 1 volume, and aniline water 2 volumes.

(8) The solution is warmed until the vapor arises and then allowed to cool for five minutes.

(9) Blot, and immerse in 30 per cent acetic acid one minute.

(10) Blot, plunge in absolute alcohol until but little more stain comes off, clear in xylol, and mount in balsam.

A useful modification is given by Meves and Duesberg (1908). Successful Benda preparations are excellent. The mitochondria are stained a deep violet color against a rose background. They are also much more permanent than Altmann preparations. Unfortunately the method is long, tedious, and difficult. It has been much employed in the study of spermatogenesis.

c. Champy-Kull's (1913) aniline-fuchsin, toluidin blue and aurantia. This is a modification of the methods of Altmann and Benda:

(1) Champy's fluid:

3 per cent potassium bichromate	7 c.c.
1 per cent chromic acid	7 c.c.
2 per cent osmic acid	4 c.c.

Twenty-four hours.

(2) Wash in aq. dest., then a mixture of 1 part acetic acid pyrolignosum rect. and 2 parts 1 per cent chromic acid, twenty hours.

(3) Wash in aq. dest. thirty minutes and mordant in 3 per cent potassium bichromate, three days.

(4) Wash in running water twenty-four hours, dehydrate, clear, imbed and section.

(5) Altmann's aniline fuchsin (10 gm. acid fuchsin to 100 c.c. aniline water) heating quietly over flame.

(6) Allow to cool six minutes, pour off stain, wash rapidly in aq. dest.

(7) Counterstain with 0.5 per cent toluidin blue one to two minutes. Rinse in aq. dest.

(8) 0.5 per cent aurantia in 70 per cent alcohol, twenty to forty seconds.

(9) Differentiate in 95 per cent alcohol, dehydrate, clear and mount.

The nuclei are colored blue, the mitochondria red, the ground substance greenish yellow. It is especially recommended by Gatenby for invertebrates.

d. Regaud (1910) has used the iron-hematoxylin method of Heidenhain after a large variety of fixatives, the best of which is his formalin and bichromate mixture.

(1) 3 per cent potassium bichromate 20 c.c., formalin 5 c.c., for four days, changing every day.

(2) Mordant in 3 per cent bichromate for seven days, changing every second day.

(3) Wash in running water twenty-four hours, dehydrate, clear, imbed, and section as indicated.

(4) Pass sections down to water as indicated.

(5) Mordant in 5 per cent iron alum at 35°c. for twenty-four hours. Rinse in aq. dest.

(6) Stain for twenty-four hours in hematoxylin made up as follows: Dissolve 1 gm. pure crystals of hematoxylin in 10 c.c. of absolute alcohol and add 10 c.c. of glycerin and 80 c.c. of distilled water. A few weeks should be allowed for the stain to ripen. When ready for use it can be employed over and over again for about ten times. The traces of iron alum added to the stain are helpful. The crucial point in the technique is this passing from the mordant to hematoxylin. The slides must be rinsed in *distilled* water, otherwise the iron alum will form a dense black precipitate in the stain. On the other hand, if they are rinsed too much, all the iron alum mordant will be removed. It is necessary to strike the happy mean in which a darkening of the hematoxylin alone occurs. It is always difficult to get good hematoxylin, and it is best to keep on hand a ripe alcoholic solution.

(7) Differentiate in 5 per cent iron alum under microscope, wash in tap water half an hour, dehydrate, clear and mount.

This is the most permanent as well as the simplest of all mitochondrial stains. It may be used in the damp climates of most of our marine biological laboratories, where the Altmann method and its modifications are useless. It is advised that the beginner try it with an organ like the pancreas. Unfortunately the fixation rarely gives good results with embryonic tissues; for these the older osmic acid-containing fixatives are best adapted. It is often possible to make use of material fixed in the usual way with formalin by starting out with step (2). Moreover, the preparations can be counterstained in a variety of ways (Cowdry, 1916). The coloration is less specific than that obtained by modifications of the Altmann method. Many cytoplasmic granulations which are not mitochondria are, like the mitochondria, colored blue-black.

e. Murray's method for mitochondria and bacteria. This is a modification of the preceding technique.

(1) Formol-Müller. (Potassium bichromate 2.5 gm., sodium sulphate 1 gm., distilled water 100 c.c., formalin 10 c.c.) over night.

(2) Mordant in Müller's fluid two to seven days.

(3) Wash in running water, dehydrate, clear and imbed in paraffin as indicated. Cut sections 5 μ.

(4) Remove paraffin, pass to water.

(5) 3.5 per cent iron alum at 50°c., fifteen minutes.

(6) 0.5 per cent aq. hematoxylin at 50°c., fifteen minutes.

(7) Differentiate with similar iron alum solution. Dehydrate, clear and mount in balsam. In this way both mitochondria and bacteria are colored bluish black. If the sections are decolorized with 0.5 per cent hydrochloric acid in 70 per cent alcohol the bacteria alone remain colored.

Bacteria are often well stained also by the various modifications of Altmann's method. When the differentiator is methyl green they are colored green, when toluidin or methylene blue, they are blue; while the mitochondria are stained crimson with the fuchsin. The root nodules of clover afford satisfactory material because they are easily obtained and invariably contain readily stainable intracellular bacteria and mitochondria.

f. Dubreuil's (1913) iron hematoxylin method for blood cells:

(1) Take up the fluid to be examined in a pipette containing several times its volume of 0.5 to 1 per cent osmic acid. Transfer to a centrifuge tube. A good fixation is obtained in about an hour. Then add aq. dest., centrifuge, and decant. The blood cells remaining are shaken up with absolute alcohol and then passed into a weak solution of celloidin. A drop is allowed to spread on a slide, which, before complete desiccation, is plunged into 80 per cent alcohol.

(2) The mitochondria in the cells are then stained with iron hematoxylin, as indicated above.

g. Bensley's copper chrome hematoxylin method. By this method the mitochondria are colored blue against a yellowish brown background.

(1) Either Altmann's osmic bichromate mixture (p. 199) or in Bensley's acetic osmic-bichromate fluid (p. 199), twelve to twenty-four hours.

(2) Wash, dehydrate, clear, imbed, and section.

(3) Pass down to water. Saturated aqueous copper acetate, five minutes.

(4) Wash in several changes distilled water, one minute, 0.5 per cent hematoxylin, one minute. If the copper acetate has not been sufficiently washed out, a black precipitate forms in the hematoxylin. The hematoxylin should be well ripened. It may be obtained by dilution down from a 10 per cent alcoholic stock solution.

(5) Rinse in aq. dest. 5 per cent neutral potassium chromate, one minute. The sections should turn a dark blue-black color. If they are only a light-blue shade, rinse in aq. dest., place again in the copper acetate, and carry through as just described several times until no increase in color results.

(6) Wash in aq. dest. and return for a few seconds to the copper acetate in order to convert all the dye into the copper lake.

(7) Wash in aq. dest.

(8) Differentiate under the microscope in Weigert's borax-ferricyanide mixture (borax 1 gm., potassium ferricyanide 1.25 gm., and aq. dest. 100 c.c.) diluted with 2 volumes of aq. dest.

(9) Wash six to eight hours in tap water.

(10) Dehydrate, clear, and mount in balsam.

h. Bensley's (1911) neutral safranin method:

(1) 2.5 per cent potassium bichromate 100 c.c., mercuric chloride 5 gm., for twenty-four hours.

(2) Wash, dehydrate, clear, imbed, and section.

(3) Preparation of stain: Add slowly sat. aq. sol. of the color acid, acid violet, to sat. aq. sol. of the color-base, safranin O, contained in a flask until a precipitate no longer

forms. The point of neutralization may be roughly determined by dropping a little of the mixture on filter-paper from time to time until the outside red ring of safranin disappears and the whole blot takes on a neutral color. Filter. The filtrate should be as nearly as possible colorless. Dry the precipitate on filter-paper for twelve hours, collect it, and make a saturated solution of it in absolute alcohol.

(4) Pass sections down through two changes of toluol and absolute alcohol in order to remove all traces of paraffin or toluol, which might interfere with the staining. Then through 95, 70, and 50 per cent to aq. dest. (Chrome- and osmium-fixed material must be bleached in permanganate and oxalic acid, and sublimate-fixed tissues must be treated with Lugol's iodine solution for about ten seconds and washed in aq. dest.).

(5) Dilute the alcoholic stock solution of the dye with an equal volume of aq. dest. and stain for from five minutes to two hours.

(6) Blot quickly with several layers of filter paper.

(7) Plunge into pure acetone and pass immediately to toluol without waiting to drain.

(8) Examine under the oil immersion and if necessary differentiate in oil of cloves. If this is not sufficient, the slide, after rinsing in absolute alcohol, may be instantaneously flooded with 95 per cent alcohol, and then passed back through absolute alcohol to toluol.

(9) Wash in two changes of toluol and mount in balsam.

Working on the same principle, a number of stains can be made up for mitochondria (Cowdry, 1913). Note also Bensley's neutral gentian method. These methods were devised by Bensley chiefly to aid in the study of the mitochondria and other cytoplasmic constitutents in the pancreas.

i. Bensley's brazilin-wasserblau method for the mitochondria and secretion antecendents of the thyroid gland (1916).

(1) Zenker's fluid, less acetic acid, plus 10 per cent formalin, twenty-four hours.

(2) Wash, dehydrate, clear, imbed, and section.

(3) Pass down to water.

(4) Iodize with Lugol's solution, thirty seconds.

(5) Stain in following solution several hours: Phosphotungstic acid, 1 gm.; aq. dest., 100 c.c.; brazilin, 0.05 gm. The brazilin is first dissolved in a small quantity of distilled water by the aid of heat and added to the phosphotungstic acid solution. Ripening may be accelerated by the addition of 0.4 c.c. of hydrogen peroxide, or of a few drops of a solution of soluble molybdic acid. The solution deteriorates with age and should not be used after three days.

(6) Rinse in aq. dest. and place for one to five minutes in phosphotungstic acid, 1 gm. wasserblau, 0.2 gm.; aq. dest., 100 c.c.

(7) Wash rapidly in water, dehydrate in absolute alcohol, clear in toluol, and mount in balsam.

j. Meves's (1905) new Victoria green method: This method is intended for red blood cells which are simply stained in the fresh condition by the addition of a 4 per cent iodic-acid solution to which a small quantity of new Victoria green (malachite green) has been added.

k. The methods of silver reduction employed by many Italian investigators are essentially modifications of the original method of Golgi (p. 344). They undoubtedly reveal mitochondria in most cases, but one would hesitate to attribute any high degree of specificity to them.

4. Experimental Error in Mitochondrial Technique. Mechanical injury to the cells by the use of forceps during removal, before fixation, must be

avoided. Allowing a surface film of the tissue to dry in air as it stands on the autopsy table will alter the whole appearance of the contained mitochondria. Osmotic changes are likewise harmful. Merely keeping the tissue in salt solution is detrimental. If the tissues cannot be fixed absolutely fresh, they should be set aside in a cool place and the surface layers should be removed with a razor just before preservation.

The various ingredients of the fixations have different powers of penetration. In respect to the most superficial cells they act simultaneously and give good preservation. As one passes inward their influence is successive and various types of artifact are often produced.

Before reaching any conclusions as to mitochondrial alterations in experimental conditions, it is essential to make sure that all the mitochondria have been preserved and that they retain the form exhibited during life. The number of mitochondria may appear to be reduced after faulty fixation, incomplete mordanting and excessive differentiation of the stain. Mitochondria are never increased in amount through technical errors. The most common change in the mitochondria brought about by fixation is a segmentation or rounding up of rods and filaments into spherules. Consequently the experimenter will wish to assure himself that this alteration is not taking place by comparison with living unstained and supravitally colored cells. The reverse alteration, of a lengthening of the mitochondria, never results from mistakes in technique.

Special methods for the quantitative estimation of mitochondria have recently been devised (Du Noüy and Cowdry, 1927,* and Cowdry and Covell, 1927†).

II. The Golgi Apparatus (Reticular Apparatus Bennennetz‡)

While there is so little agreement as to just what the Golgi apparatus is, it is difficult to describe the technique for its demonstration. What may, however, be regarded as the "type structure" was first revealed by Golgi (1898) in nerve cells through fixation in a mixture containing potassium bichromate and osmic acid followed by impregnation with silver. The apparatus appears jet black against a yellowish background. It is a conspicuous structure consisting of an intricate network of anastomosing strands. This network may closely envelop the nucleus, be concentrated to one side of it, or else be scattered rather diffusely throughout the cytoplasm.

In 1902 Kopsch showed that the same material can be blackened by prolonged treatment with 2 per cent osmic acid. On this affinity for both silver and osmium all the modern methods for revealing the Golgi apparatus

* Du Noüy, P. L., and Cowdry, E. V., *Anat. Record*, 1927, xxxiv, 313.

† Cowdry, E. V., and Covell, W. P., *Anat. Record*, 1927, xxxiv.

‡ For a discussion of the relation of the Canalicular apparatus, Trophospongium Säftkanalchen etc., to the Golgi apparatus see: Cowdry, E. V., General Cytology, Chicago, 1924.

are based. Few cytological reactions are more fickle and inconstant, but, when after many attempts the technique is successful, convincing and very beautiful preparations result.

Unlike the mitochondria, the Golgi apparatus cannot be studied unstained or supravitally colored in the living cell with any degree of satisfaction except perhaps in some plants. For a summary of advances in this direction, and for much original work, including the supravital coloration of the apparatus with neutral red in saprolegnia, see the monograph of Guilliermond.* Parat and his associates, in a brilliant series of studies, have advanced the view that the material which we recognize as the Golgi apparatus in animal cells which have been impregnated with silver or with osmium, is represented in the living cell by droplets which may be colored with neutral red. We await a final proof of this hypothesis with eagerness.

With both silver and osmium methods considerable experimentation is necessary in order to obtain the best results. The factors to be varied are principally the composition of the fixative and impregnating substance and the time during which they are allowed to act. During impregnation it is always advisable to keep the tissues in the dark and instructions as to temperature requirements should be carefully followed. When either the silver nitrate or osmic acid becomes blackened it should be renewed. It is important for the beginner to start with the most favorable material. The spinal ganglion cells of young mammals such as the rabbit are perhaps the best for this purpose. The acinous cells of the pancreas are also recommended but are somewhat more difficult to handle. All of the methods of impregnation outlined below frequently bring to light the mitochondria also.

1. Silver Methods.

a. Cajal's (1912) uranium nitrate silver method.† This is one of many methods devised by Cajal. It is recommended for embryos and young animals.

(1) Uranium nitrate 1 gm., formalin 15 c.c., and aq. dest. 100 c.c. eight to twenty-four hours.
(2) Wash quickly in aq. dest.
(3) 1.5 per cent silver nitrate twenty-four to forty-eight hours.
(4) Rinse in aq. dest.
(5) Hydrochinon 2 gm., formalin 6 c.c., aq. dest. 100 c.c., anhydrous sodium sulphate 0.15 gm., twelve hours.
(6) Wash in aq. dest., dehydrate quickly, clear, imbed and section.

b. Da Fano's (1920)‡ cobalt nitrate silver method. Here the uranium nitrate is replaced by cobalt nitrate. In other respects the technique is

* Guilliermond, A. *Arch. d'Anat. Micr.*, 1927, xxiii, 1.
† Many useful hints are given by Carleton, H. H., *J. Roy. Micr. Soc.* 1919, p. 321.
‡ Da Fano, C., *J. Roy. Micr. Sci.*, 1920, p. 157.

similar. Da Fano has, however, so carefully attempted to control troublesome experimental conditions that the various steps are given in detail.

(1) Fix in cobalt nitrate 1 gm., aq. dest. 100 c.c., formalin 15 c.c. six to eight hours. The formalin need not be neutralized unless it is strongly acid. In the case of embryos and delicate tissues, when shrinkage is to be feared, reduce the formalin to as little as 6 c.c. With cartilage and small pieces less than 3 mm. thick, like the organs of mice, shorten the time of fixation to three to four hours. Hollow organs, such as the stomach and intestine, are better filled with the fixing fluid for one hour and then cut into pieces of convenient size and shape. For the spinal cord, cerebellum and cerebrum of adults, eight to ten hours is recommended, but fixation should never exceed twenty-four hours. In the case of the testicle, he advises injection of the fixative through the aorta and then immersion in it.

(2) Wash quickly in aq. dest. and impregnate in 1.5 per cent silver nitrate twenty-four to forty-eight hours. The concentration of silver nitrate should be reduced to 1 per cent for very small fragments easily permeable, and be increased to 2 per cent for tissues containing much fat and for the spinal cord. Impregnation is effected at room temperature in a majority of cases. When difficulty is experienced in impregnation the use of an incubator at 36° to 37°c. is advised.

(3) Wash rapidly in aq. dest. and cut down the tissues again to a thickness of 2 mm. or less.

(4) Reduce in Cajal's mixture, above mentioned, twelve to twenty-four hours.

(5) Wash in aq. dest. one-half hour. Cut with a freezing microtome or imbed in paraffin. The Golgi apparatus should be colored dark brown or black against a yellow background. The preparations may be made more permanent by gold toning.

(6) Pass to water. Then 0.1 to 0.2 per cent gold chloride, two hours.

(7) Counterstain with alum carmine, dehydrate, clear and mount.

2. Osmium Methods.

a. Kopsch's Method. Immersion of small pieces of tissues in 2 per cent osmic acid for eight to sixteen days often brings to light the Golgi apparatus but there is considerable shrinkage and the tissues become rather brittle.

b. Sjovall's (1905) modification:

(1) 10 per cent formalin, eight hours.

(2) Wash in aq. dest.

(3) 2 per cent osmic acid at 35°c., two days.

(4) Dehydrate, clear, imbed.

c. Hirschler's (1918) modification:

(1) Saturated aqueous mercuric chloride 10 c.c., 2 per cent osmic 10 c.c., at room temperature one to three hours.

(2) Wash in running water then in aq. dest., one half hour.

(3) Two per cent osmic acid at 25°c., twelve to sixteen days.

(4) Wash twenty-four hours in running water, dehydrate, clear in chloroform and imbed.

d. Kolatchew's method (Nassonov, 1924):

(1) 3 per cent potassium bichromate 10 c.c., 1 per cent chromic acid 10 c.c., and 2 per cent osmic acid 5 c.c., twenty-four hours.

(2) Wash in running water twenty-four hours.

(3) 2 per cent osmic acid 40°c., eight hours, three to five days at 35°c.

(4) Wash in aq. dest., dehydrate, clear and imbed.

e. Weigert's Mann-Kopsch method as modified by Gatenby:*

(1) Mann osmio-sublimate mixture (sat. aq. corrosive sublimate in salt sol., 10 c.c., 1 per cent osmic acid, 10 c.c.) one quarter to three hours or more.
(2) Wash in aq. dest. fifteen to thirty minutes.
(3) 2 per cent osmic acid, room temperature ten to fourteen days.
(4) Wash in running water two hours or more.
(5) Dehydrate, clear, and imbed.

The Golgi apparatus is blackened and the mitochondria and ground substance are colored reddish brown. Gatenby suggests as subsequent treatment:

(*a*) "The blackening may be extracted step by step in turpentine, and the appearance of the cell granules studied at intervals.

(*b*) "If the mitochondria are not stained black by the OsO_4, one may proceed directly to the Altmann method (but preferably after cautious treatment in 0.125 per cent permanganate of potash).

(*c*) "The nuclear structures may be stained in safranin, crystal violet, or acid fuchsin. The sections are brought down to distilled water and transferred to watery solutions of the dye. A few minutes generally suffice to stain the nuclei."

Many suggestive experiments have been made with the Mann-Kopsch method by Ludford.† He has found that the time of fixation bears but little relation to the degree of impregnation with osmic acid, and that better impregnation is obtained when the temperature of the osmic bath is increased above that of the room. With progressive rise in temperature the following sequence of changes was observed:

(*a*) "The apparatus appears first as granules or faint rodlets.

(*b*) "The rodlets become thicker and the cytoplasm commences to shrink.

(*c*) "The rodlets appear to be anastomosed to form a network, and the ground substance becomes coarsely granular.

(*d*) "The whole of the Golgi apparatus is impregnated deep black, and the ground cytoplasm is distorted so as to give the appearance of a tangled network, or reticular structure, and there is considerable non-specific reduction of the osmic acid.

(*e*) "Thereafter, the cell becomes more and more deeply osmicated, until the cytoplasm appears homogeneously black."

Cells which have thus become very black may be bleached by dilute solutions of potassium permanganate, by hydrogen peroxide and by nascent chloride. Ludford prefers the last. In view of these experiments he says the "Golgi apparatus is that region of the cytoplasm of cells which brings

* Lee's Microtomist's Vade-mecum. Ed. 9. Ed. by Gatenby, J. B., and Cowdry, E. V., London, 1928.

† Ludford, R. J., *J. Roy. Micr. Soc.*, 1924, p. 269.

about the reduction of osmium tetroxide at a lower temperature, or in a shorter time, than is required to produce a total blackening of the cell."

3. **Experimental Error in Revealing the Golgi Apparatus.** Some investigators prefer to impregnate the Golgi apparatus with silver and others with osmium. Which is the least open to objection it is difficult to say. The precautions to be observed are in many respects similar to those mentioned under the heading of "Mitochondria," p. 204. The influence of variations in temperature has not been so carefully studied with silver as with osmium. Before placing any reliance in the Golgi apparatus as an indicator of cellular activity it is essential to make sure that the technique being used brings to light all the Golgi apparatus, not only a part of it. The surface and volume of this peculiar structure have recently been measured quantitatively by means of a special technique in spinal ganglion cells.*

* Covell, W. P. *Anat. Record,* 1927, xxxv, 149.

CHAPTER VI

EMBRYOLOGICAL METHODS

C. E. McClung and Ezra Allen

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I. Introduction

The range of embryological methods is extensive and their character varied, so that in a book of this sort it would be impossible to treat them exhaustively. Especially is this true if the practices of experimental embryology are involved. The only consideration that the latter will receive will be in connection with the treatment of micromanipulations. The main emphasis here will be placed upon the technique for fixed and sectioned material.

II. Securing Embryological Material

Unlike most other materials, those used in embryological work may not be obtainable just when wanted, so that it becomes necessary to take into consideration the seasonal occurrence of developmental stages in animals and to follow the rule of getting the material when it is available rather than when it is wanted. Because of the large numbers of animal forms and their variable embryological behavior it will be possible here to consider only types and these very briefly. If one desires information with regard to the various forms available for embryological studies he should consult works devoted to comparative embryology. It will be sufficient here to mention that many aquatic invertebrate forms, both fresh water and marine, offer convenient sources of material. Certain snails may be kept in aquaria and will there provide at intervals batches of eggs which may be traced through the various developmental processes. Fish often are convenient for this purpose. The eggs are stripped from the female and the milt from the male, and after fertilization the successive steps of cleavage and somatogenesis may be obtained.

Amphibian material is usually available only in the spring, but if secured at this time may be obtained in large quantities. Frogs and toads are most often the source of this material, but certain Urodeles also serve. Axolotols are conveniently kept in aquaria and in the spring lay a number of batches of eggs which are very excellent embryological material.

One of the most extensively studied embryological forms is the chick. It has certain advantages, one of which is the ease with which material may be obtained. All stages of development may be secured by timing the periods of incubation, either under a hen or in an incubator. Mammalian mate-

rial is very difficult to handle although its source is more continuous and convenient than any other. The commonest forms utilized for this purpose are the mouse, rat, rabbit and pig.

Knowing the reproductive cycle of any animal it is not a matter of great difficulty to obtain a complete series of embryological stages by appropriate timing. In the case of the pig the most convenient method is to go to an abattoir and there secure uteri, collecting a large number of stages which later may be measured and sorted. Because of its availability in this manner the pig has been much used, although it has certain structures which are unusually developed. Later stages of development are not hard to obtain but the early ones offer many more difficulties. As a means for securing these in the case of the mouse, Allen offers the following directions:

Mouse Embryos. Fixation of small mammalian embryos *in situ* frequently results in distortion through contraction of the uterine muscles. The following method has avoided this result in mouse embryos from five to eight days gestation age, the critical period. Remove both horns of the uterus by severing the tubes, the mesometrium, and the vagina. Transfer to Locke's solution in a small dish with paraffin-covered bottom. Stretch the horns and pin fast, ventral side up, pinning through the vagina or bladder, the tubes, and the mesometrium. Under a low power binocular remove the muscle from the exposed surface, at least over each capsule (*decidua capsularis*). This may be done by starting at the vagina and carefully dissecting the muscle as a sheet, or removing it in short strips. The capsules may now partly be freed with ease from the underlying muscle and left attached at the mesometrial end. If carefully done no pressure is exerted upon the capsules. The fixative desired may now be added in small quantities, pipetting it directly upon the capsules while in the Locke's solution. In about fifteen minutes, after several applications of the fixing fluid, the pins may be removed, and if no contraction takes place the horns removed to the pure fixative. Destin's fluid (see formula, Chapter IX, p. 422) has given excellent results for both normal and degenerate embryos, but is not to be relied upon for chromosome fixation. Fix from two to eight days, wash for twenty-four hours, and run to 50 per cent alcohol by gradual steps. Again pin fast and dissect the capsules sufficiently to remove one-half of the capsular tissue, watching for the appearance of the embryo. When it is uncovered, it may either be removed and further dehydrated for sectioning, or left in position and the capsule and embryo removed from the muscle and prepared for sectioning together. By staining in toto with alcohol cochineal, orientation of the embryo in either case is simple; the weak stain is readily removed from the sections by acid alcohol. If desired to treat the embryos differently, the capsules may be severed from each other after the muscle has been removed and placed in different fixatives. Bouin's and B-15 are both good fluids for preserving the capsules and embryos, but they render the decidua capsularis a little tougher than does Destin's fixative. If used, the duration of fixation should be as brief as possible, especially if dissection of the capsule is proposed. The chrom-acetic-formol fluid gives a beautifully white embryo and surrounding tissue. Mouse embryos younger than six days of gestation are too small to be seen under the binocular, and their capsules should not be dissected but sectioned whole. Capsules of six to eight day embryos may be trimmed on each side after the embryo is uncovered, thus avoiding many superfluous sections. This trimming should be done before passing to the clearing fluid, but not before they have been in 70 per cent alcohol long enough to harden fairly well.

Mouse Uteri for Blood Vessels. For this purpose, either ligate the blood vessels before removing the uterus from the body, or stretch the two horns in the body cavity and cover with Locke's solution. To this add the mixture previously described (p. 184) ae B-20.

After the horns have stiffened, remove them to the pure fixative and let remain an hour. The fixative may be either warm or at room temperature. Carry to 70 per cent alcohol by gradual steps. When thus treated, the blood tends to retain its color for some time and the vessels stand out against the yellow color of the rest of the tissue.

III. Study of Living Material in Normal and Isotonic Media

In embryological work it is fortunately the case that the entire history of a single organism may be continuously observed under appropriate conditions. The normal picture of development thus secured is extremely helpful in the interpretation of sectioned material later, and wherever possible should always be carried out. The technical processes involved here are extremely simple and require no elaboration. However, when it comes to the experimental modification of normal processes we enter a field of great difficulty and diversity.

Much of the early development of a chick embryo may be observed by producing a window in the shell of the egg. This is best accomplished by using a hard rubber ring of about three quarters of an inch in diameter. The outline of this is marked on the side of the shell with a pencil and within this the shell is thinned by means of a file. The rubber ring is then cemented onto the shell by the use of shellac or a collodion solution. After this is hardened the shell and membrane within the ring are removed and the space filled up with albumen from another egg. Over this is placed a cover glass which soon becomes firmly attached by the drying of the excess albumen. The egg is then put into an incubator and by turning the window to the upper side of the egg the embryo may be observed at any time and its development traced.

There are two general means for carrying out experimental modifications. The first is by chemical agents and the second by physical or mechanical methods. Because of the great diversity of chemical modifications of development and their special character it is not expedient to discuss them at length here, and those interested are referred to such works as those of Jacques Loeb and others. In recent years mechanical methods for modifying embryological processes have been highly developed and have served to advance our knowledge of normal processes materially. These also are of extreme diversity but because they have not been so fully published the methods will be discussed somewhat at length by Chambers in the chapter on fresh material.

IV. Prepared Material

1. General Character of Processes. The methods employed for embryological study do not differ materially from similar ones in cytological and histological work. The same fixatives that produce good results on cells and tissues also act best when the entire body of an embryo is to be preserved. Likewise the methods of sectioning in paraffin and collodion

are essentially the same as elsewhere employed and do not require repetition here.

a. Whole Mounts of Fixed Specimens. It happens, because of the small size of embryos, that they are often studied entire and so we have special methods for mounting and studying entire organisms rather than their parts. Chick embryos are conveniently mounted entire, very much like a stretched preparation. Since these are so often used for embryological studies the method of their preparation will be given fully.

It is best to begin with an embryo of about thirty-six hours' incubation since it is of convenient size and of sufficient development to show the different systems. Commonly the directions require the removal of the embryo from the shell of the egg, but better results are obtained by fixing before removal and the method described will follow that plan.

Remove the egg from the incubator and with a sharp instrument like a scalpel, grasped near the point with the fingers so that it cannot enter very far, pierce the shell on the side. Avoid making this opening on the top because the embryo floats upwards and may be injured. After the shell is broached in this manner the opening may be enlarged by carefully removing pieces of the shell with forceps until it is about 1 inch in diameter. The later operations are best continued with the opening uppermost.

After the shell and its membrane have been taken off, a small quantity of albumen is withdrawn by a pipette so that a depression above the embryo is produced. Into this is pipetted a quantity of the fixing fluid. The best one for this purpose is formol-nitric (p. 422). One of the picro-formol-acetic mixtures may also be used in a similar manner. By withdrawing this first quantity of fixative and some of the coagulated albumen, fresh fixative may be brought very intimately into contact with the embryo. After fifteen or twenty minutes the embryo is sufficiently fixed and may then be removed for further treatment. The best way to do this is to take a pair of fine scissors, curved on the side, and with them carefully cut around the outside of the sinus terminalis. Take an appropriate sized section lifter and insert it into the yolk beneath the embryo and carefully lift up the blastodisc. Transfer this to a Syracuse watch glass and by means of a pipette so agitate the water that the yolk is completely removed from the blastodisc. When this has been accomplished, withdraw the water and yolk and carefully spread the blastodisc until it is free from wrinkles. On the embryo thus extended drop a small quantity of Worcester's fluid (p. 420). This completes the fixation and at the same time hardens the blastodisc so that it is much more resistant to after-treatment. Allow the Worcester's fluid to act for ten to fifteen minutes and then treat the object as a section fixed in a sublimate fluid (p. 421), finally mounting in balsam. The usual dehydrating and clearing fluids may be used but most substances used for clearing, like xylol, cause extensive shrinkage, as much as 33 per cent. An extensive series of experiments has revealed the fact that of all the clearing fluids available the synthetic oil of cassia, or cinnamic aldehyde, causes the least shrinkage. By careful dehydration and clearing it is possible to secure a blastodisc in which practically no shrinkage occurs after fixation. This external lack of distortion is the evidence of a similar normal condition within the embryo and the tissues will be found almost free from the separation which usually occurs.

Embryos up to seventy-two hours may be prepared in this manner if care is taken to support the cover glass in the case of older specimens. Owing to the thickness of the damar exposed at the edge of the cover it is advantageous to ring the covers with some protecting cement (p. 472, Cement).

By a process of microinjection (Chambers, p. 40) such embryos may have their circulatory system filled with India ink and when mounted

entire make beautiful preparations. Older embryos may be injected in the same manner. These are not mounted like an ordinary microscopical preparation but are cleared with methyl salicylate and placed in convenient containers for observation.

In the study of the developing osseous system, embryos may be rendered transparent by treating them without previous fixation with a 1 per cent solution of potassium hydroxide for twenty-four hours. They are subsequently preserved in glycerin.

b. Dissected Specimens. Dissections of embryos, even of small size, are entirely feasible and, in connection with sections of similar embryos, afford a valuable means of correlation. Heuser has developed very refined methods for such microdissections. He fastens the fixed embryo to a small piece of ground glass with gelatin. After the embryo has become attached it is then dissected under alcohol with the aid of the binocular microscope. In this manner he has been able to trace out even the finest connections of nerves. For some purposes it is better to stain the embryo entire before beginning the dissection and for this purpose alum cochineal is perhaps the best stain.

c. Sectioned Specimens. While cleared preparations and dissections are very helpful in reaching an understanding of embryonic structures, final resort in most cases must be made to the study of serial sections. Here, as elsewhere, the preservation of the material in its normal form is of the highest importance. Formerly Zenker's fluid was much recommended as a fixative, but it causes extensive shrinkage and now resort is made almost entirely to the picro-formol-acetic combinations. Picric acid in connection with sulphuric acid in the formula of Kleinenberg has long been used but seems to be inferior to the picro-formol-acetic mixtures (p. 425).

All of the usual precautions with regard to gradual transfer of the specimen from one fluid to the other hold with somewhat added emphasis in the case of embryos. The methods of staining are similar to those used in histological practice with the exception that for most purposes the advantages lie with in toto staining. For this purpose no better combination has been evolved than the alum cochineal stain (p. 468).

For a complete understanding of the structure of a bilaterally symmetrical body such as a vertebrate embryo it is necessary to have embryos of similar stages of development cut in three planes at right angles to each other. Two of these series are longitudinal sections, one passing parallel with the plane which divides the body into right and left halves, and the other at right angles to this. In addition to these two longitudinal sections, one series cut at right angles to them and passing transversely through the body is necessary. These sections, when cut by the paraffin method, come off in a continuous ribbon which is then subdivided and mounted as slides in such manner that by proceeding from left to right successively in one slide after the other, the entire embryo is passed through

in order, emerging at the last section on the lower right hand corner of the last slide. The sections are therefore read like the letters in the words of the lines of a printed page. It is obvious that with the necessity for this continuous series of sections, every precaution must be employed in sectioning and in subsequent treatment, to avoid the loss or mutilation of any section or series of sections. Also for ready passage from one section to the other they should be arranged in absolutely straight rows and so placed on the slide that sections at right angles to the rows fall also in lines. A thoroughly good embryological slide will show the sections therefore uniformly spaced and in straight rows.

For small embryos the ordinary size of glass slip, 1 × 3 inches, may be used, but larger specimens are better mounted upon slips 1½ × 3 inches, or 2 × 3 inches.

The thickness of sections depends upon the purpose in view. Ordinarily the best thickness is about 10μ, and it is very desirable to have a microtome which will cut a practically uniform series of sections. This is of particular merit when reconstructions are to be made.

It is always important to secure complete spreading of the sections, but it is of unusual value in the case of embryological sections because if they are to be used for reconstructions any distortion due to unequal or incomplete spreading will seriously interfere with the production of the model.

The experienced worker, having at his command sections of an embryonic stage in the three dimensions, will be able to reconstruct in his mind the general configuration of organs and their relations to each other. However for a permanent record, resort is often had to the process of reconstruction. This consists essentially in making drawings or photographs of sections at regular intervals and from these making transfer to wax plates which are of such a thickness as to correspond in magnification to the two dimensions represented in the section. These wax plates are then cut out and piled one upon the other until they reproduce in their essential form the structure of the entire embryo. Of course, due to many technical difficulties, the contour of the surfaces is not a continuous one and resort is had to a method of smoothing by the use of heated implements until the normal outline is restored.

Dissections and whole embryos are often very helpful in determining the exact amount of reduction or addition necessary to restore the correct outline. There are many details involved in the process of reconstruction which cannot be given here, but for which information may be secured by consulting such works as Minot's laboratory manual.*

* Minot, C. S. Laboratory Text Book of Embryology. Ed. 2, Phila., 1910.

CHAPTER VII

HISTOLOGICAL METHODS

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METHODS OF STUDYING RED BLOOD CELLS

Raphael Isaacs

Counting red blood cells 216. Counting white blood cells 220. Counting blood platelets 221. Estimating hemoglobin percentage 223. Measure of hemoglobin percentage by means of oxygen combining power of blood 224. Color index 228. Determining volume of blood cells 229. Obtaining volume index 229. Washing red blood cells 229. Determination of specific gravity of blood 230. Determining resistance of red blood cells to hypotonic salt solutions 231. Determining resistance of red blood cells to heat 232. Determining sedimentary rate of red blood cells (suspension-stability) 232. Measuring red blood cells 234. Studying hemagglutination and hemolysis of red blood cells 235. Studying sickling of red blood cells 239. Demonstrating special features or structures in red blood cells 239. Blood platelet diluting solutions 241.

I. Methods of Counting Red Blood Cells

1. Apparatus and Materials. *Solution*, Hayem's, Toisson's or Gower's, freshly filtered (p. 242).

Alcohol, 70 per cent, for washing the skin.

Blood Lancet. A pen point may be used or a Hagedorn needle. There are special types of blood lancets on the market, some with guards to measure the depth to which the needle penetrates the skin, and others, automatic, with a point released by a spring.

Red Blood-counting Pipette. It is well to use one with a Bureau of Standards certificate or one which has been checked or calibrated, so that its accuracy is known. The best type of blood-counting pipette is one with a blunt tip as this is less likely to break. There are some special types of blood-counting pipettes made to overcome certain defects of the ones commonly used. In the Trenner diluting pipette the inner end of the measuring capillary tube terminates slightly above the lower level of the mixing chamber and at right angles to its floor. Blood drawn into this tube runs by capillary action to the end of the tube which is, therefore, automatic in this respect. This tube is graduated differently from the original Thoma diluting pipette in that the total volume of the capillary tube is $\frac{1}{200}$ of the content of the bulb of the red counting pipette (for the white counting pipette the capillary tube volume is $\frac{1}{20}$ of the volume of the mixing chamber). This pipette eliminates one error in measuring the blood

for dilution. The Piney pipette* is more elaborate and is useful for very exact measurements. The Shaweker pipette has the dilutions engraved on the capillary stem instead of the figures 0.5 or 1.0 as in the Thoma pipette.

Counting Chambers. There are several types. The Bürker† type is now becoming more popular and gradually replacing the older type with a round moat and round center table. The newer counting chambers are made of one piece of glass and should be chosen in preference to those in which the ruled surface or the supporting surfaces are cemented to the glass slip. There are numerous types of rulings. Many of them are of historical and evolutionary interest. The simplest and most practical is the Neubauer ruling in the improved form.‡ The directions given here will enable one to use any type of ruling.

2. Technique. The blood may be taken from the ball of the finger or the lobe of the ear. The lobe of the ear is less sensitive and the patient cannot see the manipulation. In a dog the blood can be taken from a razor cut on the margin of the ear; in a rabbit a slight cut over one of the ear veins will give sufficient blood; in a mouse the tip of the tail may be cut off and in a rat a slight cut in the tail may be used.

The place may be rubbed lightly with 70 per cent alcohol and the alcohol allowed to evaporate completely. The blood lancet should be sterile and free from old clots of blood, as these tend to make the instrument appear dull. If the instrument is chisel shaped and has a wide sharp point, a free flow of blood is obtained with the least possible manipulation.

The bottle of diluting solution should be open and easily available so that no time will be lost. The mouth piece of the pipette is held in the mouth and the lancet is plunged into the skin. The blood must flow freely. The first drop of blood is usually wiped away and with the least possible pressure a second drop is allowed to form. When a drop about 3 mm. in diameter has accumulated, the tip of the pipette, which must be chemically clean and absolutely dry, should be touched to it and the blood drawn up to the mark 0.5. If the blood is very dilute, as in a patient with profound anemia, the blood may be drawn up to the 1.0 mark. This will mean that after the final mixing it will be diluted 1 to 100 instead of 1 to 200. This must be taken into account in making the final calculation. The pipette should be held at about 45° with the point downward. Great care should be used to have the blood reach the exact line. Excess blood is wiped from the tip of the pipette and with the pressure maintained by holding the cheeks steady, the tip of the pipette is put into the diluting solution. By suction the solution is drawn up and the pipette is snapped with the finger once or twice to release the glass bead which sometimes adheres to the wall and retains an

* Piney, A. *Lancet*, 1924, i, 906.

† Bürker, K. *Arch. f. d. ges. Physiol.*, 1907, cxviii, 460.

‡ Neubauer, E. *J. Lab. & Clin. Med.*, 1924, x, 56. *J. Am. Med. Assn.*, 1925, lxxxiv, 947.

air bubble beneath it. The diluted fluid is drawn up to the mark 101, the pipette is withdrawn from the solution and the finger is immediately placed over the tip. The mouth piece is then removed from the mouth and the pipette is shaken for at least three minutes. The pipette should be shaken in such a way that all the blood will be thoroughly mixed.

A simple revolving in one direction or shaking in the longitudinal axis is usually not sufficient to include the blood which is held in the capillary ends of the diluting chamber, and motion should preferably be in several directions. Automatic shakers have been described. The suspension should be absolutely uniform with no visible clots. If the process is delayed at any stage or the manipulation is not rapidly performed, the blood may clot. The hemacytometer slide should be ready so that the drop of diluted blood can be put under the cover glass. The cover glass and slide must be grease-free. As with the pipettes, the slides and covers should be of known accuracy, preferably with a U. S. Bureau of Standards certificate. These are best cleaned with a little alcohol, acetone or ether. If the slide is of the old type and has balsam underneath the ruled surfaces, these solvents can be used only on the cover glass. There are two kinds of hemacytometer slides in use, those with a round moat and round table which are filled before the cover glass is put on, and those with a rectangular table which are filled after the cover glass is put on. The latter type of slide is of two kinds, one with smooth glass ridges to support the cover glass and one with ground glass ridges. When a smooth glass type is used the cover glass is placed on the ridges and slight pressure exerted until Newton's colored rings (rainbow effect) can be seen where the glass surfaces come in contact. With the ground glass type the cover glass is merely put into place. Some workers place a microscopic drop of water on the ground glass surface to hold the cover glass in place. This should never be large enough to spread over the entire surface. When a cover glass which has been checked by the Bureau of Standards is used, the mark etched in the glass should be on the upper surface. Great care should be used not to disturb the cover glasses after they are once in position. Some types of hemacytometer slides are provided with clips to hold the cover glass in place. The pipette with the diluted blood is well shaken and if it has been allowed to stand any length of time, it should be shaken at least three minutes before it is used. After the blood is thoroughly mixed, one or two drops of solution are blown out to expel the portion which has been in the capillary part of the pipette. The mouth piece is held in the mouth and a drop of blood is blown underneath the cover glass until the space above the table is filled. The drop must be put under the cover glass with one sweep and must not be allowed to proceed in little waves, as the distribution of cells will then be uneven. If bubbles form underneath the cover glass, it indicates that the glassware was not grease-free and the manipulations must be repeated after cleaning the slide and the cover glass. Then with slight suction any

excess of fluid is quickly removed so that the side moats are not flooded. With the older type, round moat slides, a drop, the size of which is determined by trial, is put on the top of the table and the cover glass is placed over this. This second type has the advantage that with it evaporation is prevented, but the rectangular type is more easily filled.

The cells are allowed to settle for at least two minutes and then are counted. The low power objective (16 mm.) and No. 10 ocular are used for counting. If one is not familiar with the appearance of the cells he may use the 4 mm. objective (high-dry). The slide is moved until the smallest squares are in the field. Some slides are provided with a deeply ruled groove which points to the fine rulings used in counting the cells. This is of aid to some in finding the area to be counted. Great care must be used to have the slide absolutely level while the cells are settling and the slide must never be jarred or the cover glass moved after the blood suspension has been placed on the slide.

In counting the cells each worker should adopt some system with reference to the cells falling on lines so that no duplication will be made in the counting. Probably the simplest method is to include all cells falling on lines above and to the left of a square in the total count for that square, and to reject all cells falling on the line to the right and at the bottom of the square, as these will be included when the other squares are counted. Squares may be counted in blocks of 25 (5×5) or in blocks of 16 (4×4) and blocks may be selected running across or diagonally down the field. In some types of ruling, the blocks are separated, for convenience in counting, by double or triple ruled lines on the edges of the blocks. In others, a single line is ruled through the middle of the set of small squares which form the border of the blocks. In the former type of ruling, the double or triple ruled lines should be regarded as a single line in allocating the corpuscles which touch them. In the second type of ruling the line does not form a boundary in itself, and therefore does not enter as a factor when the cells on the edge are counted.

Automatic tally counters are sometimes of help in enumerating the cells.

Each of the smallest squares in the center of the ruled area measures $\frac{1}{400}$ sq. mm. in area and is $\frac{1}{10}$ mm. deep. Under ideal conditions the cells which have sedimented in 400 of these squares should be counted, representing a volume of 1 sq. mm. in area and $\frac{1}{10}$ mm. in depth. If the cells in any smaller number of squares are counted, calculation must be made to make up the total; thus if the cells of 100 small squares are counted the number obtained will have to be multiplied by 4 to obtain the number in a square millimeter area. The greater the number of squares counted, the less the inaccuracy. In a specimen of normal blood with an average of 5,000,000 cells per cubic millimeter, 100 of the smallest squares will contain 625 cells, if the dilution is 1 to 200. Care should be taken to exclude the

white blood corpuscles in counting the red cells. The former can be recognized easily by their difference in refractivity. Occasionally a few are counted with the red cells, producing a small error in the final calculation.

An example of a blood count is as follows:

Number of red cells in 100 small squares = 625.

Since there are 400 squares in 1 sq. mm., this number must be multiplied by 4 to obtain the number in a square millimeter. As the column of fluid is $\frac{1}{10}$ mm. deep, to obtain the number in a cubic millimeter this number must be multiplied by 10. Since the dilution was $\frac{1}{2}$ unit of blood diluted to 101 units, the suspension of corpuscles was $\frac{1}{200}$ the concentration of the original blood. Therefore, the number of cells in $\frac{1}{10}$ cu. mm. of the diluted suspension multiplied by 200 will give the number of cells per cubic millimeter of blood.

$625 \times 4 \times 10 \times 200 = 5{,}000{,}000$ red blood corpuscles per cubic millimeter of whole blood.

The chief sources of error are improper measurements in making the dilutions and unequal distribution of cells on the hemacytometer; the latter may be prevented to some extent by thoroughly mixing the blood suspension before it is put on the hemacytometer slide, by allowing the blood to rush under the cover glass with one sweep instead of little waves, by using great care to prevent jarring of the slide or movement of the cover glass, and by having the cover glass and slide absolutely grease-free. An experienced operator usually must count on an average error of ± 5 per cent in his red cell counts, which would mean that counts which vary by 250,000 when the absolute count is around 5,000,000 can be considered identical. When the double type of hemacytometer is used, the count can easily be made in duplicate. If a pipette with the diluted blood has stood for some time and is reshaken, the counts are not quite as accurate as when the freshly made suspension is used.

The pipette should be cleaned at once, using water to wash out the blood, then alcohol and finally ether. The alcohol and ether should be sucked through. A hand bulb or a suction apparatus may be used. It is well to have a horse hair as part of the equipment to clean out particles which become adherent to the inside of the tube. If the blood coagulates in the pipette, it should be cleaned out at once. If particles adhere to the sides of the tube they may be digested with an acid solution of pepsin. The slide and cover glass should be washed with water and the ruled area should be carefully dried with soft lens paper.

II. Method of Counting White Blood Cells

1. Apparatus and Materials. The method is similar to that used in making a red blood cell count, but as a diluting solution 1 per cent acetic acid is used. The pipette is calibrated so that a dilution of 1 to 10 or 1 to 20 may be obtained.

2. Technique. The blood is collected and drawn into the white blood cell counting pipette to the 0.5 mark for ordinary blood and to the 1.0 mark when the white count is very low. The diluting solution is then drawn in until the mixture reaches the 11.0 mark, using the same technique as with the red blood cells (q. v.). The same precautions are used in mixing and in placing the mixture on the slide. The cells in each of the four corners of the ruled area are counted. With the ordinary ruling, 1 sq. mm. just fits into the low power field (16 mm. objective, No. 10 ocular). With the usual rulings this space is divided into sixteen squares. The cells in a minimum of 4 sq. mm. should be counted for each enumeration. Those cells falling on the lines which form the left border and the upper border of the field should be included with the cells in that area. Those falling on the lines which form the right border and the lower border should be included with the other squares.

The calculations are as follows:

Number of cells in each square millimeter area multiplied by 10 gives the number of cells in a cubic millimeter; multiplied by the dilution 20, when the blood is drawn to the 0.5 mark and by 10 when the blood is drawn to the 1.0 mark, gives the number of cells per cubic millimeter of the undiluted blood.

Example:
Number of cells per square millimeter, 45.
Dilution 20 times.
$45 \times 10 \times 20 = 9000$ white blood cells per cubic millimeter of whole blood.

III. Methods of Counting Blood Platelets

1. Methods of Ottenberg and Rosenthal, Wright and Kinnicutt, Rees and Ecker, and Kristenson.* Using the technique described under "Counting Red Blood Cells" (p. 216) and with all the glassware chemically clean, the blood is drawn from a freely flowing drop to the 1.0 mark in a red blood counting pipette and as a diluting solution the sodium citrate solution of Ottenberg and Rosenthal, or the solution of Wright and Kinnicutt or Rees and Ecker or of Kristenson may be used. The diluting solution (freshly filtered) and the blood are drawn up to the 101 mark. The blood is thoroughly mixed in the pipette and one or two drops are blown out and discarded. A drop of the diluted mixture is then allowed to run underneath the cover glass of a hemacytometer slide. This is covered with a small glass or bell jar to prevent evaporation and allowed to stand for ten minutes. If desired, the preparations (except those with the Wright and Kinnicutt and Kristenson solutions, which hemolyze the red blood cells) may be used during this time to make a red blood cell count. The

* Ottenberg, R. and Rosenthal, N. *J. Am. Med. Assn.*, 1917, lxix, 999.
Wright, J. H. and Kinnicutt, R. *J. Am. Med. Assn.*, 1911, lvi, 1457.
Rees, H. M. and Ecker, E. E. *J. Am. Med. Assn.*, 1923, lxxx, 621.
Kristenson, A. *Acta Med. Scandinav.*, 1922, lvii, 301.

platelets are counted under the high dry objective. The normal counts with these methods vary from 200,000 to 400,000. The calculations are the same as those for a red blood cell count (q. v.) the dilution being 100 times instead of 200.

Example:
Number of platelets in 1 sq. mm. area (400 of the small squares) $\frac{1}{10}$ mm. deep, 250
In 1 cu. mm. $10 \times 250 = 2500$
Dilution 100 times
$2500 \times 100 = 250{,}000$ per cubic millimeter of blood.

2. Method of Buckman and Hallisey.* Venous blood is drawn into a paraffined tube. The blood is then drawn into a red blood counting pipette to the 0.5 mark and the special diluting solution of Buckman and Hallisey is drawn in until the mixture reaches the 101 mark. The pipette is shaken for three minutes and after blowing out and discarding a few drops, some of the suspension is put under the cover glass of a hemacytometer slide. After allowing three minutes for the cells to settle, a red blood cell count is made in the usual way. (See p. 216, Method of Counting Red Blood Cells.) In five minutes a white blood cell count may be made from the preparation. After twenty minutes the platelets are counted, using the high dry objective. Cells in 4 square millimeters are counted. The calculations are as given under Method of Counting Red Blood Cells (p. 216). With this method counts may be made as long as four hours after the specimen is taken. The normal counts vary from 246,000 to 328,000 platelets per cubic millimeter, with an average of 284,000 per cubic millimeter. The platelets appear as discrete, uniformly distributed, pale blue, oval bodies about $\frac{1}{6}$ to $\frac{1}{3}$ the size of normal red blood cells. The cytoplasm of the cells appears finely granular and the periphery of the platelets slightly irregular.

3. Method of Pratt.† A few cubic centimeters of the special diluting solution of Pratt are placed in an absolutely grease-free watch glass. A drop of the fluid is taken up in a sterilized platinum loop (3 mm. in diameter) and is brought into contact with a freshly flowing drop of blood from a puncture wound in the ear. There should be three or more parts of fluid to each part of blood. The mixture is placed on a grease-free slide and covered with a thin cover glass. If desired the dilution may be made on the slide. The mixture should be allowed to spread so that the red blood cells are well separated. It is best to make two preparations. The red blood cell count is made from another drop of blood in the usual way, using the standard methods for this purpose. (See p. 216, Method of Counting Red Blood Cells.) The ratio of the number of blood platelets to the number of red blood cells is calculated from an enumeration of both elements in the

* Buckman, T. E. and Hallisey, J. E. *J. Am. Med. Assn.* 1921, lxxvi, 427.
† Pratt, J. H. *J. Am. Med. Assn.*, 1905, xlv, 1999.

fresh preparation. The oil immersion lens is used. A diaphragm with square opening, placed in the ocular, or an Ehrlich eyepiece may help in making the count. From 250 to 500 red blood cells should be counted in two preparations. The normal platelet count with this method varies from 226,000 to 725,000, with an average of 469,000 per cubic millimeter of blood.

IV. Methods of Estimating Hemoglobin Percentage

1. Sahli Method.* Into the graduated tube supplied with the Sahli apparatus, $\frac{1}{10}$ normal hydrochloric acid is placed until it reaches the mark 10. The blood is then drawn into the special hemoglobinometer pipette until the mark 20 cu. mm. is reached. A fairly large-sized drop of blood is needed. The tube is held vertically and the hemoglobinometer pipette is lowered into the tube until its tip is just beneath the surface of the hydrochloric acid. The blood is blown slowly into the solution until the pipette is practically empty. Then the hydrochloric acid is sucked up and gently blown out several times until all the blood is washed out. In this way bubbles are avoided and no blood is lost. At the end of exactly sixty seconds water is added and gently mixed with the blood and hydrochloric acid solution, using a fine, bead-tipped glass rod. Water is added drop by drop and the solutions mixed, and the color is compared with the standard tubes which accompany the apparatus. When enough water has been added to make the color of the blood mixture and that of the standard similar, the height of the column of fluid is read and the number is the percentage of the hemoglobin. The solid glass standard tubes have the advantage over liquid hematin hydrochloride suspensions inasmuch as the color of the latter tends to fade. The Sahli pipette is cleaned with water and then with alcohol and ether. In this method 100 per cent is equivalent to 17.3 gm. of oxyhemoglobin per 100 c.c. of blood.

2. Tallqvist Method. A drop of blood is taken up on the special blotting paper supplied with the Tallqvist books, which are on the market. After the drop of blood has just dried and the gloss is no longer visible, the blotting paper is bent so that there is a white sheet behind the drop of blood. With the paper in this position the blood stain is held opposite one of the holes in the color chart supplied with the book. The paper is then moved from hole to hole until the colors are matched. The reading then gives the percentage of hemoglobin.

3. Dare Method. The blood is taken between two plates of the blood "pipette" which is supplied with the colorimeter. This is quickly transferred to the instrument so that the glass plate is toward the observer. A yellow light (either a candle or electric light) is used and the color is compared with the standard glass plate and the latter is moved until the colors, as seen through the eye piece, appear identical. The reading is then

* Sahli, H. Diagnostic Methods, Edited by N. B. Potter, Phila., 1918, p. 749.

made on the wheel on the side of the machine, and this is taken as the percentage of hemoglobin.

4. Newcomer Method.* A special hemoglobinometer, a modified Duboscq colorimeter, is used. The instrument has two cups, one filled with plain water and the other with the blood preparation. The latter is made by diluting 10 cu. mm. of blood drawn in a special pipette supplied with the apparatus, to 50 volumes, with 5 c.c. of 1 per cent hydrochloric acid. With this dilution readings down to 40 per cent hemoglobin may be obtained, but for lower values 20 cu. mm. of blood must be drawn into the pipette. The standard used for comparison is a piece of colored glass superimposed over the cup containing the plain water. The instrument is calibrated to read in percentage of hemoglobin when the color of the hematin-hydrochloric suspension is practically at its maximum depth. On this scale100 per cent corresponds to 16.92 gm. of hemoglobin per 100 c.c. of whole blood. After the solution has stood for one-half hour, the readings are correct to within approximately 1 per cent. Readings made before this time must be corrected by the use of figures given in a table which accompanies the instrument. If the standard glass slip is not exactly 1 mm. in thickness, correction must be made for this from a table supplied with the instrument. In this method, 100 per cent is equivalent to 16.92 gm. of oxyhemoglobin per 100 c.c. of blood.

V. The Measure of Hemoglobin Percentage by Means of the Oxygen Combining Power of the Blood

1. Method of Van Slyke and Stadie, Modified by Lundsgaard and Möller.† A Van Slyke gas apparatus is cleaned and filled with mercury. Distilled water, 6 c.c., is placed in the cup and 2 to 3 drops octyl alcohol and 0.3 c.c. of a 1 per cent solution of saponin in water are added. The upper stop-cock is opened and the solution drawn down into the apparatus so that the upper level of the solution is at the upper stop-cock. This stop-cock is then closed and the mercury lowered until its level is at the lower stop-cock. This will create a reduced pressure over the water-alcohol-saponin solution and some of the contained gases will be extracted. The machine should be shaken to facilitate this process. The lower stop-cock is so turned that when the mercury is lowered still further the solution will run down into the lower bulb where it is trapped. By turning the lower stop-cock 180° the mercury is allowed to run up into the mixing bulb and capillary pipette. The air which is gathered above this is released through the upper stop-cock. The mercury is then lowered so that the capillary stem and mixing chamber are completely empty and by turning the lower

* Newcomer, H. S. *J. Biol. Chem.*, 1923, lv, 569.

† Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
Van Slyke, D. D. and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.
Lundsgaard, C. and Möller, E. *J. Biol. Chem.*, 1922, lii, 377.

stop-cock 180° the water-alcohol-saponin solution is again allowed to run into the mixing bulb. The apparatus is thoroughly shaken. The solution is then run back into the lower bulb and the air which has been released from the liquid again trapped over the mercury and expelled through the upper stop-cock by allowing the mercury to fill the mixing bulb and capillary pipette. This process must be repeated several times until no more air can be withdrawn from the fluid. The fluid is then drawn into and trapped in the lower bulb, and the mixing bulb and capillary pipette are filled with mercury.

Blood, 5 to 10 c.c., is withdrawn from a vein and mixed with 0.1 c.c. of a 20 per cent solution of sodium citrate or 0.1 c.c. of a 2 per cent solution of heparin in water. These will prevent coagulation. Blood, 3 to 5 c.c., is placed in a separatory funnel and the funnel is rotated so that the blood forms a thin film on the sides. The upper stop-cock is removed occasionally to insure saturation of the blood with oxygen. The blood will usually be saturated after about two to three minutes' exposure in this way. Of the oxygenated blood, 2 c.c. are placed in the upper cup of the gas apparatus and 2 or 3 drops of octyl alcohol placed over it. The upper stop-cock is slowly opened and the mercury level lowered until the blood is entirely within the graduated capillary stem. The mercury can be lowered until some of the supernatant octyl alcohol also enters. Several drops of mercury are put in the cup and allowed to be sucked into the capillary of the upper stop-cock. The latter is tightly closed and the mercury lowered until its upper level is at that of the lower stop-cock. The stop-cock is then turned 180° so that the contained water-octyl-alcohol-saponin solution runs into the mixing chamber and mixes with the blood. The two are shaken together and after about one-half to one minute the blood is completely laked. The mercury is then allowed to run in through the lower stop-cock until the laked blood solution again fills the graduated capillary pipette. There will be a little gas over this solution. Of a 20 per cent air-free potassium ferricyanid solution (which has been boiled and preserved under liquid petrolatum) 0.1 c.c. is carefully placed in the cup and by turning the upper stop-cock is allowed to run into the blood mixture without loss of the imprisoned air. The upper stop-cock is filled with mercury and the mercury level is lowered until it reaches that of the lower stop-cock.

The blood mixture is thoroughly shaken for at least one minute. At this stage the oxygen is released from the hemoglobin. The blood mixture is then drawn into the lower bulb and the stop-cock turned 180° and the mercury allowed to fill the mixing bulb and the capillary pipette. The mercury bulb is held against the capillary pipette so that the level of the mercury in the bulb and the level of the mercury in the pipette are the same. A little fluid is usually imprisoned over the mercury. The top of this column is used as the meniscus in reading the height of the column of gas which partly fills the graduated capillary tube. This reading is noted. The

mercury is then lowered until its level is that of the lower stop-cock. The blood mixture is then allowed to run into the mixing chamber and the shaking process repeated. It is then again imprisoned in the lower bulb and mercury is allowed to fill the mixing bulb and the graduated capillary stem. After leveling the mercury bulb with the mercury in the capillary stem the reading of the meniscus above the fluid column above the mercury is again taken. The whole process should be repeated several times until this reading becomes constant. The amount of gas imprisoned represents the oxygen liberated from the 2 c.c. of blood at the temperature and barometric pressure of the room. This gas contains the air which was mechanically dissolved by the blood as well as the oxygen which was held by the hemoglobin. From the table (Table II) the amount of air physically dissolved by the blood may be obtained and this should be subtracted from the observed reading. To obtain the hemoglobin percentage the corrected reading must then be multiplied by the factor given in Table II. With this method, 100 per cent is equivalent to 15.6 gm. of oxyhemoglobin per 100 c.c. of blood.

The apparatus may be cleaned by washing repeatedly with 5 per cent ammonia water, followed by distilled water.

TABLE I

FACTORS FOR CORRECTION FOR BAROMETRIC PRESSURE

Barometer	$\frac{B}{760}$	Barometer	$\frac{B}{760}$
732	0.963	756	0.995
734	0.966	758	0.997
736	0.968	760	1.000
738	0.971	762	1.003
740	0.974	764	1.006
742	0.976	766	1.008
744	0.979	768	1.010
746	0.981	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

2. **Method of Van Slyke and Neill.*** This method is similar to the previous one except that there are several modifications in the details of manipulating the reagents. The solution is:

Potassium ferricyanide	3.0 gm.
Saponin (Merck)	3.0 gm.
Octyl alcohol	3.0 c.c.
Water to	1000 gm.

* Van Slyke, D. D. and Neill, J. M. *J. Biol. Chem.*, 1924, lxi, 554.

TABLE II

FACTORS FOR CALCULATING RESULTS FROM ANALYSIS OF 2 C.C. OF BLOOD SATURATED WITH AIR

Room Temperature °C.	Gas Physically Dissolved by 2 c.c. of Blood. Subtract This Figure from the Observed Reading to Obtain Volume of Oxygen Liberated from the Hemoglobin c.c.	Factor by Which the Corrected Gas Volume is Multiplied to Give Percentage of Hemoglobin. (20.9 Per Cent Oxygen Corresponds to 100 Per Cent Hemoglobin in This Table) Per Cent
15	0.037	$222 \times \frac{B^*}{760}$
16	0.036	$221 \times \frac{B^*}{760}$
17	0.036	$220 \times \frac{B^*}{760}$
18	0.035	$219 \times \frac{B^*}{760}$
19	0.035	$218 \times \frac{B^*}{760}$
20	0.034	$217 \times \frac{B^*}{760}$
21	0.033	$216 \times \frac{B^*}{760}$
22	0.033	$214 \times \frac{B^*}{760}$
23	0.032	$213 \times \frac{B^*}{760}$
24	0.032	$212 \times \frac{B^*}{760}$
25	0.031	$211 \times \frac{B^*}{760}$
26	0.030	$210 \times \frac{B^*}{760}$
27	0.030	$209 \times \frac{B^*}{760}$
28	0.029	$208 \times \frac{B^*}{760}$
29	0.029	$207 \times \frac{B^*}{760}$
30	0.028	$206 \times \frac{B^*}{760}$

* B = Barometric reading.

Analysis is carried out as follows for 1 or 2 c.c. of blood: The reagent mixture is stirred by rotation in its flask to obtain an emulsion of the octyl alcohol. Into the Van Slyke gasometric apparatus (see method No. 1) 7.5 c.c. (for 2 c.c. of blood use 10 c.c.) of solution are measured from the 2 c.c. mark in the chamber to the 5.5 c.c. mark in the cup. The air is removed from the solution by evacuating the apparatus and shaking for three minutes. During the shaking the surface of the mercury should be in

the tube at the bottom of the chamber and 1 cm. below the point at which the tube broadens into the 50 c.c. bulb. Thus located, the mercury remains fairly quiet and has minimum contact with the reagent solution, so that there is but insignificant reaction between the reagents under these conditions. During the de-aeration of the reagent solution a sample of thoroughly oxygenated blood is drawn into a 1 c.c. (or 2 c.c.) pipette, preferably provided with a stop-cock and rubber tip. The three minute extraction of the reagents being complete, 6 c.c. of the solution are forced up into the cup, leaving 1.5 c.c. in the chamber. The sample of blood is at once run under the liquid in the cup into the chamber. The pipette is then withdrawn with the least possible agitation of the reagent solution in the cup and 1 c.c. of the latter is permitted to flow into the chamber, rinsing the blood in the capillary and leaving 5 c.c. in the cup. The stop-cock is then sealed with a drop of mercury and the 5 c.c. of solution in the cup are discarded. The purpose of the discarded upper 5 c.c. of solution is to protect from air the lower 1 c.c. which is returned to the chamber, thus avoiding one error in the method of determination.

The apparatus with the blood and reagents in the chamber is evacuated and shaken three minutes. Air-free 1N sodium hydroxide, 1 c.c., is placed in the cup and the carbon dioxide is absorbed by admitting 0.5 c.c. of the hydroxide into the chamber under diminished pressure, leaving the oxygen and nitrogen. Correction for the absorbed nitrogen which is fairly constant may be made (after calculation of the volume of the gases to 0°c. and 760 mm. pressure, subtract 1.36 volumes per cent) and the amount of oxygen calculated directly. From this the hemoglobin content may be determined (See Table 11).

VI. Color Index

The color index of the blood is the quotient obtained by dividing the percentage of hemoglobin (obtained from the readings of one of the hemoglobinometers) by the percentage of red blood cells, considering 5,000,000 per cubic millimeter as the normal (100 per cent). Since the red blood cell count of a normal person may vary almost 2,000,000 per cu. mm. during the course of the day* and since the hemoglobin may vary from 15 per cent to 20 per cent during the same period, and considering also that the percentage of hemoglobin and number of red blood corpuscles may be slightly lower in women than in men, the number is more or less arbitrary and to be strictly accurate must be corrected for such factors as sex, age, standard of hemoglobinometer used, diurnal variation in the hemoglobin and red blood cell count. Under ideal conditions the index would be 1; when there is a great discrepancy between the number of red blood cells and the amount of hemoglobin, the index will be below 1 when the hemo-

* Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A. *Johns Hopkins Hosp. Bull.*, 1925, xxxvii, 14.

globin *per se* is deficient, and above 1 when there is more hemoglobin in each cell than normal.

VII. Methods of Determining the Volume of Blood Cells

1. Macro Method. Blood, 10 c.c., is added to a graduated centrifuge tube containing a few crystals of sodium citrate or oxalate, or 0.1 c.c. of a 2 per cent solution of heparin. The blood is mixed thoroughly and is centrifuged at 3000 revolutions per minute for thirty minutes. The volume of corpuscles can then be read and the percentage obtained by multiplying by 10.

2. Micro Method (Van Allen). A drop of fresh blood from a lancet cut in the skin is drawn exactly to the top of the capillary part of the special hematocrit tube of Van Allen. Sodium oxalate solution (1.3 per cent) is then drawn into the tube until the bulb is about half filled. The bottom of the tube is closed with a rubber band or preferably a special clip which can be bought with the apparatus, and the tube is centrifuged for fifteen minutes at 2700 revolutions per minute. The relative volume of corpuscles is read directly in percentage from the scale.

VIII. Method of Obtaining Volume Index*

The term "volume index" is used to express the quotient obtained by dividing the percentage volume of red blood cells by the percentage number of red blood cells. The percentage volume of red blood cells is obtained by centrifuging whole blood either with or without an anticoagulant (before the blood has coagulated) in a hematocrit for three minutes at about 800 to 1000 revolutions per minute. The volume of red blood cells in normal individuals is 4.6 c.c. per 10 c.c. of blood. The formula for the volume index is:

$$\frac{\dfrac{\text{Volume of red blood cells}}{4.6}}{\dfrac{\text{Number of red blood cells per cu. mm.}}{5{,}000{,}000}}$$

In normal people this number is 1.0; in pernicious anemia it is greater than unity during the relapses, and in secondary anemias, less than unity.

IX. Method of Washing Red Blood Cells

An anticoagulant (a few crystals of sodium citrate or oxalate or 0.1 c.c. of a 2 per cent aqueous solution of heparin per 10 c.c. of blood) is added to a volume of blood and the red blood cells are sedimented by centrifugalization. The plasma is withdrawn with a pipette, and a physiologic saline solution (0.85 to 0.75 per cent sodium chloride; Ringer's solution; Locke's solution; Tyrode's solution, or their various modifications) is added to make the original volume. The corpuscles are mixed with the solution and re-sedimented by centrifugalization. The salt solution can be withdrawn and

* Haden, R. L. *Arch. Int. Med.*, 1923, xxxi, 767.

the process repeated several times. Usually 3 washings are necessary to remove all traces of plasma.

X. Method for the Determination of the Specific Gravity of the Blood

1. Method of Hammerschlag. The simplest laboratory method for determining the specific gravity of whole blood is that of Hammerschlag.* Mixtures of chloroform (sp. gr. 1.485) and benzol (sp. gr. 0.88) are made in a wide test tube. A drop of freshly drawn blood is allowed to fall into the mixture from a pipette held just over the surface of the liquid. If the drop remains stationary when suspended in the liquid, it is assumed that its specific gravity is the same as that of the liquid. If it sinks, it is heavier and more chloroform must be added and, after mixing, the solution tested with a fresh drop of blood. If the drop remains on top it is lighter and more benzol must be added. After adding either the benzol or chloroform to change the specific gravity, the fluid must be stirred thoroughly. When the proper mixture is obtained, the specific gravity of the solution is determined by the use of a hydrometer. The specific gravity of normal whole blood varies between 1.050 and 1.062. The specific gravity of plasma or serum (1.029 and 1.023) may be determined by the same method. The determinations must be carried on as rapidly as possible as the solvents used extract lecithin and cholesterol from the corpuscles and the red blood cells become distorted and hemolyzed.

2. Method of Reznikoff.† The specific gravity of the *red blood cells* is best determined by the method of Reznikoff.

Mixtures of benzyl benzoate (sp. gr. 1.115 at 20°C.) and cottonseed oil (sp. gr. 0.920 at 20°C.) are placed in separate test tubes, the first containing 5 parts each and the successive tubes containing less cottonseed oil, the last tube containing pure benzyl benzoate. These are arranged in a series of small Wassermann tubes, each containing 2 c.c. of the mixture. The range of specific gravity will be from 0.017 to 1.115. A variation of 0.02 c.c. of either fluid will correspond to a change in the specific gravity of 0.002. The red cells are prepared by centrifugalizing defibrinated blood in small Wassermann tubes for fifteen minutes at 3600 revolutions per minute. The plasma is carefully withdrawn with a pipette, leaving only the solidly packed red corpuscles. Sufficient blood can be obtained from a skin puncture in the ear or finger using a drop of 2 per cent heparin solution per 1 c.c. of blood as an anticoagulant. This can be centrifugalized in a small hematocrit tube and the corpuscles withdrawn with a capillary pipette. Drops of this thick sediment are placed in the various tubes and in the tube where the corpuscle mass remains suspended in the liquid, not rising to the top or falling to the bottom, the specific gravity is about that of the red corpuscles. The blood should be added to the oil mixture from a fine capillary pipette,

* Hammerschlag, A. *Ztschr. klin. Med.*, 1892, xx, 444.

† Reznikoff, P. *J. Exper. Med.*, 1923, xxxviii, 441.

using a rubber bulb to express the contents. A small drop of blood should be squeezed out and allowed to hang on the end of the pipette. This should then be plunged into the oil solution until it reaches about the middle of the tube. The drop can then be dislodged and it will either remain stationary, rise or sink. The specific gravity of this solution can be determined by testing a solution of similar composition with a hydrometer. The method is rapid and quite accurate for clinical purposes. The specific gravity of the normal red blood cells by this method varies from 1.092 to 1.094.

XI. Method of Determining Resistance of Red Blood Cells to Hypotonic Salt Solutions

The concentration of a solution of sodium chloride in which red blood corpuscles begin to hemolyze may be determined by the following method.

In performing this test, whole blood or washed corpuscles may be used. The blood may be drawn from a vein, but at times sufficient blood may be obtained from a lancet wound in the ear or finger. The washing process, using Ringer's solution or a similar physiologic solution, always alters the composition of the red blood cells. To wash the corpuscles, the sedimented cells, obtained by centrifugalization, are suspended in the washing solution and re-centrifugalized. This process may be repeated one or two times. The red cells are also injured during the process of centrifugalization in separating the corpuscles from the plasma. Another modification is to centrifugalize whole blood to which an anticoagulant has been added, and use the corpuscles without washing or other procedure. For comparative purposes the same method must always be used. When the whole blood is used, coagulation may be prevented by means of 0.1 c.c. of a 2 per cent aqueous solution of heparin per 10 c.c. of blood. The blood may be made into a 0.2 per cent solution of sodium citrate or oxalate by the addition of 0.2 gm. of either of these salts in powder or solution form to each 100 c.c. of blood or in this proportion. For the salt solution, 1 gm. chemically pure, dry sodium chloride is dissolved in sufficient chemically pure, neutral distilled water to make 100 c.c. The solution should have a hydrogen-ion concentration of pH 7.0. This is the stock solution. Small, chemically clean test tubes (about 70 × 10 mm.) are arranged in a rack and from two burettes salt solution or neutral distilled water are added to the tubes in varying proportions. For the preliminary test the variations from tube to tube may be made by adding 1.4 c.c. of salt solution and 0.6 c.c. of distilled water to the first tube, 1.2 c.c. of salt solution and 0.8 c.c. of the distilled water to the second tube, etc. This will give 2 c.c. of solution in each tube. The first tube will correspond to a 0.7 per cent sodium chloride solution, the second tube to a 0.6 per cent and so on. When the beginning point of hemolysis is determined, very fine gradations may be made in the strength of the salt solution between crucial points of beginning hemolysis and complete hemolysis.

A drop of the whole blood or of the corpuscle suspension is added to each tube and thorough mixture obtained by stirring with a fine glass rod, or

inverting the covered tubes. The solutions are placed in an ice box and allowed to stand until complete sedimentation of the red blood corpuscles has occurred. It is best to take the readings in about twelve hours and again in twenty-four hours. Several points are noted: first the strength of the salt solution in which the first trace of hemolysis (salt solution tinged with hemoglobin) is seen. A record is made of the first tube (highest concentration of salt solution) in which hemolysis is complete. In this tube the salt solution will be deeply red and no non-hemolyzed blood corpuscles will be seen macroscopically in the bottom of the tube. In health the minimum resistance (strength of solution in which first hemolysis is evident) is 0.47 and complete hemolysis is noted in the tube in which the strength of the salt solution is 0.30.

As a control a specimen of normal blood should always be examined at the same time as the pathologic specimen.

XII. Method of Determining Resistance of Red Blood Cells to Heat

1. Method of Isaacs, Brock and Minot.* The blood is drawn from an arm vein and mixed with sufficient crystalline sodium citrate to make a 0.2 per cent solution. Blood films are then made on cover glasses on which brilliant cresyl blue has been dried. These are counterstained with Wright's stain. These preparations are used to study the condition of the blood before it is subjected to heat. Blood, 1 c.c., is placed in a small test tube (70 × 10 mm.) and this is suspended in a water bath at a temperature ranging from 55° to 58°c. The blood of a normal individual may be used as a control. The test tube remains in the water bath for thirty minutes. It is then gently shaken to mix the blood thoroughly and blood films are made on cover glasses on which brilliant cresyl blue has been dried. In blood treated by this method, the older red cells become fragmented and hemolyzed under these conditions, whereas those showing signs of youth and a few of those which appear to be adult, remain intact. The latter are probably the younger of the adult cells. In blood in which most of the cells are young (chronic hemolytic jaundice) the bulk of the cells remain intact.

XIII. Methods of Determining the Sedimentation Rate of Red Blood Cells (Suspension-Stability)

1. Method of Fahraeus.† Blood is taken from an arm vein and placed directly in test tubes 17 cm. in length and about 9 mm. in inner diameter. The tubes contain 2 c.c. of a 2 per cent sodium citrate solution. The blood is added until the contents reach the 10 c.c. mark. The ratio of citrate to blood is 1 to 4, and the height of the citrate blood mixture is about 150 mm. The tube is inverted several times to insure thorough mixture of the con-

* Isaacs, R., Brock, B., and Minot, G. R. *J. Clin. Invest.*, 1925, i, 425.

† Fahraeus, R. *Acta Med. Scandinav.*, 1921, lv, 70.

tents and is then allowed to stand in a vertical position. The height of the clear supernatant plasma layer is measured at the end of one hour. With this method the average for healthy men is 3.3 mm. of clear plasma and 7.4 mm. for healthy non-pregnant women, during the first hour. In the newborn the average is ½ mm. per hour. During pregnancy the rate of settling of the corpuscles is more rapid, being on the average 44.9 mm. per hour. With this method, values higher than 9 mm. per hour for men and 12 mm. per hour for non-pregnant women are considered abnormal.

2. **Method of Linzenmeier.*** Blood is drawn from a vein in a 1 c.c. syringe and 0.8 c.c. placed in a special tube which contains 0.2 c.c. of a 5 per cent solution of sodium citrate. The tube measures 6½ cm. in length and the diameter is 5 mm. It is marked in two places; mark No. 1 indicates a volume of 1 c.c. and mark No. 2 is 18 mm. lower. The tube is inverted twice to insure mixing and the time is noted. The time that it takes for the corpuscles to sink from the 1 c.c. mark to the 18 mm. mark is noted and is the time used for comparison with other bloods.

3. **Method of Zeckwer and Goodell.†** Of a 3 per cent sodium citrate solution 2 c.c. are placed in a 15 c.c. centrifuge tube graduated at 0.1 c.c. intervals. Blood from the patient's vein is put into the tube up to the 10 c.c. mark. The blood and the citrate are thoroughly mixed and the tube is allowed to stand in a vertical position. The time is then recorded. The height of the column of red cells is noted at the end of one hour. The height of the plasma may be obtained by subtracting the height of the red blood corpuscles from the total height of the column of liquid. If the height of the plasma is found to be slightly above or below the 10 c.c. mark, the readings for the red blood cells must be corrected proportionately. In normal individuals the rate is 8.0 to 9.8 c.c. in the first hour.

4. **Method of May.‡** Venous blood is placed in a test tube 10 cm. in length and 6 mm. in diameter. The tubes stand in a special rack at the back of which is a cardboard scale with a gradation from 0 to 100. The blood is drawn from a vein into a syringe which has been washed with a 3.8 per cent solution of sodium citrate and which contains 0.4 c.c. of this solution. Blood, 2 c.c., is drawn into the syringe and mixed with the citrate solution. Of the citrated blood 1.8 c.c. is introduced into the special test tube. Care must be used to avoid the introduction of bubbles of air. The tube is allowed to stand for one hour in a vertical position when the height of the column of plasma above the red corpuscles is measured. With this method the cells for normal men are from 1 to 4 and for women 1 to 6. Figures up to 20 indicate a slight increase, up to 40 a medium increase, and a great increase if above this number. The blood should be taken from the patient while he is fasting.

* Linzenmeier, G. *Deutsche. med. Wchnschr.*, 1922, xlviii, 1023.

† Zeckwer, I. T., and Goodell, H. *Am. J. Med. Sc.*, 1925, clxix, 209.

‡ Piney, A. Recent Advances in Hematology, Lond., 1927, p. 261.

5. Finger Puncture Method of Cutler.* Blood, 0.5 c.c., is obtained by the puncture of the palmar surface of a finger and is collected in a small test tube, the sides of which have been wet with a 3 per cent sodium citrate solution. The blood is drawn in a special pipette 2.5 mm. in internal diameter with a stem graduated in 50 mm. divisions. The capacity of the graduated portion of the pipette is less than 0.3 c.c. A Van Allen spring attachment is used for closing the bottom of the pipette after it is filled with blood. The pipette is placed upright in a sedimentation rack and the position of the upper level of the sedimentation column of red blood cells is recorded every five minutes for one hour. This may be plotted in a graphic way using the number of millimeters as the abscissas and the time in minutes as the ordinates. If many specimens of blood are taken at one time, they may be set aside for several hours if necessary until it is convenient to start observations. When observations are to be begun the tubes are inverted several times until the corpuscles are uniformly distributed once more.

XIV. Methods of Measuring Red Blood Cells

1. Ocular Micrometer. The magnification value of the lines engraved in the ocular micrometer are determined by measuring lines, whose distance apart is known, on a stage micrometer. In the absence of the latter a hemacytometer slide may be used. A well fixed and stained blood film is placed on the stage of the microscope and the individual cells are viewed through the ocular micrometer. The slide is moved with the mechanical stage and as each cell falls under the micrometer rulings, it is measured. Usually one diameter is measured and the error caused by this factor is compensated for if sufficient cells are measured. For extremely careful work it would be necessary to measure both diameters of cells which are not round. A convenient method of recording the sizes is to check off, on a piece of paper opposite a column of figures indicating the sizes, the number of each size of red cells. It is well to measure at least 200 red cells, preferably 500, for each determination. Several areas on the field should be selected. On a film made on a glass slip, there is a tendency for the larger red cells to be gathered at the end of the film. The size of the red cells must be calculated from the number of times the ocular magnifies objects on the stage. For example, if a given red cell is covered by five lines in the ocular micrometer and it has been found that five lines will cover 6 μ on the stage micrometer, then the size of the red cell measured will be 6 μ. If the tube length is changed between observations it is necessary to re-determine the magnification. The data may be plotted on cross section paper with the abscissas representing percentage and the ordinates representing the diameter of the red blood cells in micra.

* Cutler, B. I. *Am. J. Med. Sc.*, 1927, clxxviii, 687.

There are three general types of ocular micrometers: one with a fixed scale in which 5 or 10 mm. is ruled in 0.1 mm. divisions or 0.05 mm. divisions. The second type is the movable scale micrometer which is ruled as in the first type but the scale can be moved by a screw attached to the side of the ocular. This has some advantage over the fixed scale inasmuch as a greater delicacy of movement can be obtained by adjusting the scale than by moving the mechanical stage of the microscope. The third type is the filar micrometer which combines a fixed scale and a movable line. The latter is a wire which can be moved across the field by means of a screw attached to an adjustable drum, the head of which is divided into fifty parts. This type of micrometer is used for very delicate measurements. For measurements of red blood cells it is too refined for clinical use.

2. Projection Method of Measuring Red Blood Cells. The red blood cells may be drawn on a piece of paper using the camera lucida and the magnification obtained by drawing under exactly similar conditions the lines of a micrometer slide. Another method is to project the image of the cells on a screen, using a projection microscope, and measure the cells in a similar way.

3. Photographic Method of Ponder and Millar.* This method is adapted for either fixed films or fresh blood. The blood is photographed, using a condenser working at N.A. = 1 with an objective at N.A. = 1.3. A blue light from a "Point-o-light" is used. Magnification is determined by photographing a calibrated stage micrometer at the beginning and at the end of the observations. The fixed blood film or the slide containing living blood cells under a coverslip sealed with petrolatum is photographed and the cells are measured on the developed plate. Care should be taken to secure cells which are not crenated or injured. Preparations in which there is rouleaux-formation are not suitable. A minimum of 100 cells should be measured, preferably 500 cells, although the probable error of the mean in the former case is only about twice as great as in the latter case, and with careful measurements this error is far less than the experimental error. The average number of cells per plate is about 10, so that numerous plates must be taken from different parts of the field. The cells are measured with a calibrated scale, divided into 0.1 mm., each cell being examined under a low power microscope.

XV. Methods of Studying Hemagglutination and Hemolysis of Red Blood Cells

1. Classification and Nomenclature of Blood Groups in Man. Several methods of naming the blood groups appear in the literature.

The method of Jansky† has been accepted because of priority. This is as follows:

* Ponder, E. and Millar, C. K. *Quart J. Exper. Physiol.*, 1924, ix, 67.
Dryerre, N. B., Millar, C. K., and Ponder, E. *Ibid.* 1926, xvi, 69.

† Jansky, J. *Haematol. studie u. psychotiku, Sbornik Klinicky*, 1907, viii, 85.

Group I. Serum agglutinates cells II, III and IV; cells are not agglutinable.
Group II. Serum agglutinates cells III and IV; cells are agglutinated by serums I and III.
Group III. Serum agglutinates cells II and IV; cells are agglutinated by serums I and II.
Group IV. Serum agglutinates no cells; cells are agglutinated by serums I, II and III.
Or graphically:

Corpuscles	*Serum*			
	I	II	III	IV
I	−	−	−	−
II	+	−	+	−
III	+	+	−	−
IV	+	+	+	−

+ = agglutination
− = no agglutination.

*The method of Moss** is as follows:

Group I. Serum is non-agglutinative; cells are agglutinated by serums II, III and IV.
Group II. Serum agglutinates cells I and III; cells are agglutinated by serums III and IV.
Group III. Serum agglutinates cells I and II; cells are agglutinated by serums II and IV.
Group IV. Serum agglutinates cells I, II and III; cells are non-agglutinable.

Corpuscles	*Serum*			
	I	II	III	IV
I	−	+	+	+
II	−	−	+	+
III	−	+	−	+
IV	−	−	−	−

Group I of Jansky corresponds to Group IV of Moss and Group IV of Jansky corresponds to Group I of Moss.

To avoid the error which may come from the numbers when the classification is not specified, it has been proposed† that the different groups be designated by the letters O, A, B and AB in place of Groups I, II, III and IV of the Jansky classification and Groups IV, II, III and I of the Moss system.

2. Method of Determining the Group to Which an Individual's Blood Corpuscles Belong. (Clinical.) Two stock serums which are sterile and have been kept in a cool place are needed. These are Groups II and III. Blood is drawn from the person to be tested, either from a vein or from a needle puncture of the skin of the finger or ear. Coagulation is prevented by means of sodium citrate (1 c.c. of a 20 per cent aqueous solution for 10 c.c. of blood, or 0.1 c.c. of a 2.0 per cent aqueous solution of heparin). Two separate drops of blood are placed on a slide and a drop of Group II serum is added to one drop of blood and a drop of Group III serum is added to the other drop. These are stirred separately and a piece of broken cover glass is placed on the side of each drop. Over this a grease-free cover glass is placed. This may be sealed with petrolatum or covered with a bell jar

* Moss, W. L. *Bull. Johns Hopkins Hosp.*, 1910, xxi, 63.
† Moss, W. L. *J. Am. Med. Assn.*, 1927, lxxxviii, 1921.

to prevent evaporation. The preparation should be examined during the next half hour to note if the cells are uniformly distributed or if they have become clumped into irregular masses. They sometimes become clumped into rouleaux-formations but this does not constitute agglutination for the purposes of this test. If there is no agglutination in either preparation during the course of an hour, the blood is Group I (Jansky). If both preparations show agglutination the blood is Group IV (Jansky). If only the one with Group II serum shows agglutination then the blood is Group III, whereas if only the one with Group III serum shows agglutination the blood is Group II. Agglutination is best observed under the low power of the microscope. When it is very marked it can easily be seen macroscopically. When much blood and serum are available the test may be carried out in a small test tube, using 0.5 c.c. of the serum and 0.5 c.c. of whole blood or a 5 per cent corpuscle suspension in a physiological saline solution.

3. Method of Determining Blood Group When Only Blood of Group II (Jansky) or of Group III (Jansky) is Available.* Blood is drawn from the individual to be tested and from an individual of Group II or Group III; some is allowed to clot so that the serum may be obtained and part is collected with sodium oxalate or citrate (final concentration 0.2 per cent of the anticoagulant) and the corpuscles separated from the plasma. On hollow ground slips or under cover glasses supported by pieces of broken cover glass, serum of the individual to be tested is mixed with corpuscles of the known individual, and corpuscles of the individual to be tested are mixed with serum of the known individual. When the known individual is of Group II the following table indicates the group to which the unknown individual belongs (Jansky classification).

Serum	Red Blood Corpuscles	Result			
Group II	Unknown	+	0	+	0
Unknown	Group II	0	0	+	+
Unknown is Group		I	II	III	IV

When the known individual belongs to Group III the following table indicates the Jansky group to which the unknown individual belongs.

Serum	Red Blood Corpuscles	Result			
Group III	Unknown	+	+	0	0
Unknown	Group III	0	+	0	+
Unknown is Group		I	II	III	IV

* Brem, W. V. *J. Am. Med. Assn.*, 1916, lxvi, 190.
Guthrie, C. G. and Huck, J. G. *Johns Hopkins Hosp. Bull.*, 1923, xxxiv, 37.

Individuals are occasionally found whose blood does not conform to any of the four groups.*

4. Quantitative Measurement of Hemagglutination and Hemolysis.† A drop of blood is drawn to the 0.5 mark in a leucocyte counting pipette and a sterile 0.85 per cent sodium chloride, 0.2 per cent sodium citrate solution is drawn in until the mixture reaches the 11.0 mark. The pipette is thoroughly shaken. A drop of the mixture is expelled and the rest is blown into a small test tube or vial containing a glass bead. This gives a dilution of 20 times. All glassware must be chemically clean. The freshly shaken mixture is then drawn up to the 0.2 mark of an accurately graduated red counting pipette and agglutinating serum is drawn in until the mixture reaches the 1.0 mark. The fluid is not allowed to run up into the mixing chamber of the pipette but is expelled onto a round moat type of hemacytometer slide and is thoroughly stirred with a glass rod, using care not to scratch the ruled surface. This is covered with a grease-free cover glass. To prevent evaporation the slide is placed in a level-bottomed Petri dish which contains a watch glass with water. This may be kept in an ice box or at room temperature. The number of non-agglutinated cells on the hemacytometer slide may be counted at intervals, using the high power dry objective. From these observations the rate of hemagglutination and of hemolysis per hour can be measured. With this method the maximum agglutination in human blood is reached in twenty-four hours at from 18°c. to 20°c. In dogs' blood the maximum is reached in two to four hours. Before the agglutination starts, a red blood cell count may be made from the preparation.

5. Method of Comparing Two Bloods to See if They are Compatible.‡ Sodium oxalate solution, 20 per cent is drawn to the 1.0 mark of a white blood counting pipette. Blood is then sucked in to the 1.0 mark, displacing the oxalate solution. The pipette is then filled to the 11.0 mark with normal saline. The mixture is then blown out into a small test tube. Blood is collected in this manner from both of the individuals to be compared. With a pipette (e. g., a white blood counting pipette or a fine glass tube) the following mixtures are made in a hollow ground slide, or on an ordinary glass slip which has been ringed with petrolatum:

3 drops from the first individual and 3 drops from the second individual.
2 drops from the first individual and 4 drops from the second individual.
4 drops from the first individual and 2 drops from the second individual.

These 3 dilutions may be made in duplicate. The contents of the drops are mixed with a fine glass rod and the drops are covered with a cover glass to prevent evaporation. After fifteen to thirty minutes the blood mixtures are examined for agglutination. An even distribution of the cells in all the mixtures means that the bloods are perfectly compatible. If the red cells

* Guthrie, C. G., and Huck, J. G. *Johns Hopkins Hosp. Bull.*, 1923, xxxiv, 37, 80, 128.
† Isaacs, R. *J. Immunol.*, 1924, ix, 95.
‡ Isaacs, R. Manual of Clinical and Laboratory Technic, Ed. 3, Cincinnati, 1927, p. 53.

are clumped in all the mixtures, the individuals are not compatible. If the red cells are clumped only in dilutions No. 1 and No. 2 and very few are clumped in dilution No. 3, then individual No. 2 may be used as a donor in transfusions in case of necessity, as this dilution represents a condition in which the bulk of the solution would correspond to the recipient's serum, and the lesser amount to the donor's blood. Such a condition would correspond to the giving of Group I (Jansky) blood to a patient who belongs to either Group II, III or IV.

XVI. Method of Studying Sickling of Red Blood Cells*

Fresh preparations of blood are made by bringing a clean cover slip in contact with a drop of blood from the finger tip or ear and mounting the preparations immediately on a glass slip. The edges are sealed with petroleum jelly. After standing for six hours, about ¾ of the cells will have developed the "sickle" shape or will have assumed other bizarre forms with long thread-like processes extending from the cells. After three days to six weeks the cells will re-assume the spherical form. In preparations from patients with mild symptoms 75 per cent of the cells assume the odd forms and in those from patients without symptoms 25 per cent take on the abnormal shapes. For this test, washed corpuscles suspended in physiological salt solution may be used or whole blood which has been oxalated. The results are the same in both cases. Several slide preparations should be made, as occasionally the sickling will fail to appear in one preparation. It may be well to try some preparations having one edge of the cover glass raised by a broken cover slip. This will give layers of blood cells of different thicknesses. If the cover glass is removed after the sickling has appeared, most of the cells assume the round form.

XVII. Methods of Demonstrating Special Features of Structures in Red Blood Cells*

1. Staining Red Blood Cells for Reticulum. (Permanent Preparations.)† The cover glasses are prepared as given in the technique for Wright's stain. A solution of brilliant cresyl blue (0.3 per cent) in 95 per cent alcohol (filtered) is allowed to dry on some of the cover glasses. A convenient way of applying the cresyl blue is to dip an ordinary microscope slip into the cresyl blue solution and stroke it across the cover glass and the cresyl blue will dry as an even film. The cover glass can then be polished in the usual way, using less pressure than when there is no stain on it. Blood is drawn from the finger tip as previously outlined and a drop about 2 mm. in diameter is taken on one end of a fresh cover glass. The edge of this cover glass is brought into contact with a cover glass on which cresyl blue has been dried, and the glasses are separated, and brought together in hinge fashion to mix

* Huck, J. G. *Johns Hopkins Hosp. Bull.*, 1923, xxxiv, 335.

† Cunningham, J. H. *Arch. Int. Med.*, 1920, xxvi, 405.

the cresyl blue with the blood. The cover glasses are then allowed to touch and after the blood has spread they are rapidly drawn apart. After the films have dried they are counterstained with Wright's stain, using the usual technique. In the final washing the process is not as prolonged as with the Wright stain only, as some of the cresyl blue may be dissolved out of the cells.

With this stain the red cells appear as with the usual Wright's stain technique but the reticulated cells show a blue precipitate of different patterns, depending upon the concentration of the so-called reticulum substances in the cells.

2. **Criteria for Determining the Relative Amount of Hemoglobin in the Cells Using a Stained Blood Film.** In a film stained with Wright's stain, the amount of red in the nuclei of the white blood cells is noted. If the red blood cells are faintly stained while the white cells have taken up a considerable amount of the eosin element of the stain, the red blood cells are *hypochromic*. If they have taken up a very great amount comparatively, they are *hyperchromic*. The normal condition, *orthochromic*, is best determined by the study of the staining qualities of the red blood cells of a healthy individual. The presence of a light area in the center of the cell does not necessarily mean hypochromia. Many parts of the blood film should always be studied before conclusions are drawn.

3. **Malarial Parasites.** The parasites are best studied in a drop of fresh blood mounted under a petrolatum ringed cover glass. They are recognized in the red blood cells by their granules, which are highly refractive and in active movement. The parasites stain well with the ordinary Romanowsky type of stains. For some cases fairly thick blood films are advantageous.

4. **Diffuse Basophilia, Polychromatophilia.** Romanowsky type of stains (eosin-methylene blue combinations, Wright's, Leishman's, Jenner's stains). The basophilic red blood cells stain grayish or slightly more blue than the normal red blood cells. Occasionally the distribution of the color is irregular in the corpuscle and both reddish and bluish areas are present (polychromatophilia). Both manifestations are evidence of youth in the corpuscles. The bluish staining substance is probably the same substance which precipitates as a reticulum with brilliant cresyl blue used supravitally.

5. **Stippling.** The stippling of red blood cells shows as fine bluish black dots in the corpuscles with Wright's and similar stains. The spots can be demonstrated with methylene blue alone, and are best seen in thick blood films. The method of Aub and his colleagues* is as follows:

Fix thin cover glass films of blood with methyl alcohol, and allow to dry. The stain used is methylene blue, 1 gm.; potassium carbonate, 1 gm.; in 100 c.c. of distilled water. This is the stock solution. A dilution of one part of the stain and 14 parts of water is placed over the blood for fifteen minutes. The excess stain is washed off with water until the blood film appears bluish green. The film is dried and the blood is examined using

* Aub, J. C., Fairhall, L. T., Minot, G. R. and Reznikoff, P. *Medicine*, 1925, iv, 1.

the oil immersion lens. The stippled cells are easily located. They are somewhat more polychromatic than the normal, light greenish blue erythrocytes. The basophilic granules appear dark blue, as do the nuclei of the leucocytes.

6. Nuclei, Nuclear Particles, Howell-Jolly Bodies. These structures are well shown with all the commonly used blood stains. They all take on the characteristic color of the nuclei of the other nucleated cells.

7. Refractive Granule. This structure* can be easily seen in about 1 per cent of the red blood corpuscles of normal human beings and mammals (increased in number under some conditions) in fresh blood, or fresh blood with a supravital stain, in unstained dried films, or in films stained with any of the commonly used stains. The granule is about 0.5 micron in diameter, highly refractive and does not stain. It appears as colorless or black, depending on the focus, or it may be tinged with the same or complementary color of the surrounding solution or stain. The granule is an evidence of youth and probably represents a stage in maturation of the red blood corpuscle between that of the reticulocyte and the adult corpuscle.

8. Cabot's Rings. These stain as red filaments (circular or figure eight) with most of the Romanowsky stains. The color of the red blood corpuscle included inside of the ring is the same as that outside of this structure, differentiating it from some artifacts which may cause confusion in identifying it.

XVIII. Blood Platelet Diluting Solutions

1. Buckman and Hallisey.†

Glucose	6.0	gm.
Sodium citrate	0.4	gm.
Distilled water	100	c.c.

Filter and add 0.02 gm. of toluene red (dimethyldiamidotoluphenazin). To this is added 0.1 gm. of crystal violet. The mixture is gently heated to 60°C. and held at this temperature for five minutes and allowed to cool to room temperature. It is then centrifugalized for ten minutes at 2000 revolutions per minute. The supernatant liquid must be filtered twice through three thicknesses of dry filter paper (No. 30 Whatman). This solution is preserved by 0.2 c.c. of formaldehyde solution. It keeps indefinitely.

2. Kristenson.‡

Urea	10	gm.
Sodium citrate	2.5	gm.
Corrosive sublimate	0.005	gm.
Brilliant cresyl blue	0.5	gm.
Distilled water	500	c.c.

* Isaacs, R. *Anat. Rec.*, 1925, xxix, 299.

† Buckman, T. E., and Hallisey, J. E. *J. Am. Med. Assn.*, 1921, lxxvi, 427.

‡ Kristenson, A. *Acta Med. Scandinav.*, 1922, lvii, 301.

3. Ottenberg and Rosenthal.*

Sodium citrate	3 gm.
Distilled water to 100 c.c.	

Cresyl blue 1 to 500 or methyl violet 1 to 500 may be added freshly to the solution just before using, and the solution then filtered.

4. Pratt.†

Sodium metaphosphate	2.0	gm.
Sodium chloride	0.9	gm.
Distilled water	100	c.c.

5. Rees and Ecker.‡

Sodium citrate	3.8	gm.
Distilled water	100	c.c.
Formaldehyde solution	0.2	c.c.
Brilliant cresyl blue	0.1	c.c.

6. Wright and Kinnicutt.§

Solution I. Brilliant cresyl blue	1.0	gm.
Distilled water	300.0	c.c.

The growth of yeast may be prevented by keeping the solution in an ice box.

Solution II. Potassium cyanide	1.0	gm.
Distilled water	1400.0	c.c.

The solutions are kept in separate bottles and just before using, two parts of the brilliant cresyl blue solution are mixed with three parts of the potassium cyanide solution and the fluid filtered.

7. Toisson.

Distilled water	160	c.c.
Glycerin, neutral	30	c.c.
Sodium sulphate	8	gm.
Sodium chloride	1	gm.
Methyl violet	0.025	gm.

8. Hayem.‖

Distilled water	200	c.c.
Sodium chloride	1	gm.
Sodium sulphate	5	gm.
Mercuric chloride	0.5	gm.

9. Gower.

Sodium sulphate	7	gm.
Acetic acid	4	c.c.
Distilled water to 120 c.c.		

* Ottenberg, R., and Rosenthal, N. *J. Am. Med. Assn.*, 1917, lxix, 999.
† Pratt, J. H. *J. Am. Med. Assn.*, 1905, xlv, 1999.
‡ Rees, H. M., and Ecker, E. E. *J. Am. Med. Assn.*, 1923, lxxx, 621.
§ Wright, J. H. and Kinnicutt, R. *J. Am. Med. Assn.*, 1911, lvi, 1457.
‖ Hayem, G. *Arch. de Physiologie*, 1878, v, 692.

METHODS FOR THE STUDY OF LEUCOCYTES

ETHEL M. SLIDER AND HAL DOWNEY

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A. METHODS FOR OBTAINING BLOOD*

Human blood is best taken from a finger. A lobe of the ear may be used, but in that case work is less likely to be speedy and uniform. Naegeli† recommends that the entire hand be immersed in warm water until suffused and then dried with chafing, in order to secure a free flow of blood and thus an even distribution of cells. In working with infants the great toe or the heel is used. Rabbits bleed freely if a puncture is made in the ear, preferably through the marginal vein. The ear should be shaved. In animals which are excited the vein may be contracted so that little blood flows, but a dilation of the vein may be caused instantly by wiping the spot with xylene. Guinea pigs, also, may be bled from the ear; but in this case a simple puncture may not be sufficient, and it is usually better to cut a slit in the ear. When it is necessary to secure much blood or to study that which is not peripheral, a method of ventricular puncture may be used, puncturing the heart with a paraffined or oiled needle. This is easily done on the larger animals: dogs, rabbits, and even guinea pigs. The easiest method with rats is to cut off a piece of the tail. With salamanders, also, the tail is used, and it should be well scrubbed just before taking the blood in order to get rid of the mucus. Blood taken in this way is mixed with a small amount of tissue fluid. It should be kept from contact with torn tissue or with the skin as much as possible. If the life of the animal can be sacrificed, purer blood can be obtained by opening the chest and taking it from the heart with a needle. In the case of birds a wing vein in the axillary region may be used, or, in such birds as the pigeon, the tibial vein in the ankle. A method is given by Nolf for obtaining blood from fishes without letting it touch the skin. Hold the fish in a vertical position with the head down and cut off the tail back of the dorsal fin. Run a glass cannula coated with paraffin into the dorsal aorta. Then place the fish in a horizontal position, and the blood will run out. A more usual method is to take it from the heart. The method of getting blood directly from the heart with a lip pipette is useful for embryos and small animals. It is the best method for frogs. The lip pipette described by Fry‡ is a glass pipette with one end drawn to a suitable fine-

* Methods for counting leucocytes and for supravital staining will be found on pp. 220, 84.

† Schridde, H. and Naegeli, O. Die Hämatologische Technik. Jena, 1921.

‡ Fry, H. J. *Anat. Rec.*, 1927, xxxiv, 245.

ness and fitted at the other end with a rubber tube with a glass mouthpiece. If the end of the tube is of capillary fineness so that the heart is not torn, blood will remain unclotted for a few minutes. As compared with a syringe needle it has the advantage of cheapness since the capillary tubes can be used freely and then thrown away. A glass tube is not successful unless it is of capillary fineness. A more extended account of methods for securing blood from various kinds of animals is given by Jolly.*

B. DRY SMEARS

I. Preparation

The preparation of smears is described in Part I (p. 30). Leucocytes in bone marrow, glands, or organs are best preserved by lightly touching a fresh cut surface of the tissue to a slip or cover glass or by dragging a small piece over the glass, and then drying rapidly by vigorously waving the preparation in the air. Peritoneal fluid smears are best made by the cover glass method described previously. Blood films should be fairly thin in order to demonstrate granules, especially azure or neutrophile granules. According to most authors a blood film can be more successfully stained about twenty-four hours after it has been made than when perfectly fresh. It should not, as a rule, be older than one week, although the time within which good results may be secured varies according to conditions, possibly atmospheric. Fixation and staining may be accomplished in one series of operations if an alcoholic stain is used, or the fixation may be done first, followed by a staining process.

II. Fixation and Staining Methods

1. The Romanowsky Stains. Most blood stains contain methylene blue, methylene azure, methylene violet and eosin. They are discussed at length by MacNeal.† Since Romanowsky was the first to point out the selective action of such stains, mixtures containing these dyes are often spoken of as Romanowsky stains and the red lavender color produced by them in the nuclei of lymphocytes and monocytes is known as the Romanowsky effect. There are a number of modifications. Leishman's and Wright's are similar, both being eosinates of polychromed methylene blue. Giemsa's modification contains the dyes called azure II-eosin and azure II; but since azure II is a mixture in equal parts of methylene azure and methylene blue, the stain is really a mixture of the eosinates of methylene blue and methylene azure. These stains contain methylene violet only as an impurity of the methylene blue. MacNeal prepared a stain containing methylene blue, methylene violet, methylene azure A and eosin, which he

* Jolly, J. Traité d'Hématologie. Vol. I, Paris, 1923.

† MacNeal, W. J. *J. Infect. Dis.*, 1906, iii, 412.

called "tetrachrome." It can be used instead of Leishman's stain. Pappenheim's modification* is called "panchrome" and contains the following dyes: methylene blue, toluidin blue, azure 1 (methylene azure), methylene violet and eosin. Pappenheim gives several percentage formulas for making it. It can be obtained from the Grübler Company, but the proportions which that company uses for it are not known. These stains are sold both as powders and as solutions. They are the chief Romanowsky stains in use at the present time.

2. **Wright's Blood Stain.** *a. Method of Use.* Wright's blood stain may be used on any dry smear. The mixture fixes and stains at the same time. For convenience in handling, the stain can be confined to a part of the slide by marking across one end with a piece of soft paraffin. The slip or cover glass should be level. In working with cover glasses it is well to support them by cover glass holders or simply on the ends of small corks. By means of a pipette the preparation is quickly flooded with enough stain to cover it well. A 22 mm. cover requires 8 or 10 drops, a slide 30 or 40 drops. The stain is left on for sixty seconds. Then with a second pipette distilled water is added drop by drop until a greenish metallic scum begins to form. This usually happens when the distilled water equals about half the amount of stain. The slide should be moved gently while adding the water in order to insure rapid mixing of the water and stain. The diluted stain is left on for two and one-half to three minutes. Then the preparation is plunged into a jar of distilled water, and transferred to fresh distilled water for about three minutes or until the red cells are pink and proper differentiation is obtained. Finally it is carefully blotted dry with smooth filter paper. For uniform results the final washing with water should be checked by a rapid glance at the preparation under a microscope, because different bloods require different times, and bone marrow needs an exceedingly brief wash. Variations in the staining solution also make this advisable. A preparation may be dried and examined at leisure, and then, if it is found to be too dark, it can be further washed with distilled water; but if it has been washed until too light, first class results cannot be obtained by restaining it. Nuclear structures and the azurophil granules of platelets, lymphocytes and monocytes should be distinct. When thoroughly dry the preparation can be mounted with neutral balsam or with damar.

b. Preparation of Stain and Use of Buffer Solution. A number of other points should be kept in mind in working with this stain. Although the sixty seconds' interval of concentrated stain is fixed, the rest of the time schedule may be slightly varied to suit the particular batch of stain and the kind of preparation. If the stain is obtained as a powder, both the Grübler and the American products can be made up in the proportion of 0.3 gm. to 100 c.c. of methyl alcohol. The solution should be shaken occasionally and is ready for use in three or four days. Detailed directions for making

* Pappenheim, A. *Folia haematol.*, Archiv, 1911, xi, 194.

the powder are given by Wright.* The solution is not indefinitely stable, however, as it may grow more concentrated by evaporation of the alcohol and require dilution, or contamination with moisture may precipitate it or make it liable to precipitation on the slide, or it may undergo slow oxidative changes. In the first case the time schedule may be lengthened, while with the other changes it should be shortened. Fresh distilled water is necessary for good results. It should be slightly acid and according to Haden† it may have a pH of 6.0 to 6.6. This is easily tested with phenol red or brom thymol blue. If it stands for a week or so in an ordinary glass bottle, it usually becomes unfit for use. Buffer solutions may be used instead, however, and are stable. The McJunkin-Haden buffer is satisfactory and has a pH of 6.4. It is made according to the following formula:

Monobasic potassium phosphate	6.63 gm.
Anhydrous dibasic sodium phosphate	2.56 gm.
Distilled water	1 liter

This solution can be used all through the technique of blood staining to replace distilled water in diluting the stain.

3. Giemsa. *a. After Methyl Alcohol Fixation.* (1) Fixation. For ordinary dry smears the simplest fixation is alcohol. Most directions call for a three minute methyl alcohol fixation of fresh smears and a two minute fixation of smears twenty-four hours old. This should be done in a covered dish, and the smear should be taken out and allowed to dry before it is stained. As described later on, May-Grünwald may be used instead of pure alcohol, and it has been found that the fixation given by this stain is a little finer and more uniform than that in alcohol alone.

(2) Giemsa. The alcohol fixation is followed by Giemsa's stain for fifteen minutes, after which the smear is washed with distilled water until properly differentiated and then blotted dry. Giemsa's stain should be prepared immediately before use in the proportion of 1 drop of the stock solution to 1 c.c. of distilled water or buffer. Giemsa stock solution:

Azure II-eosin	0.6 gm.
Azure II	0.16 gm.
Methyl alcohol, pure and free from acetone	50.00 c.c.
Glycerin, pure and free from water	50.00 c.c.

Dissolve the dyes in the glycerin at 60°c. and then add the alcohol warmed to 60°c. A muddy bluish color in the cells is caused by too great an alkalinity. After about twenty minutes the stain begins to precipitate. With Giemsa's stain and its modifications it is well to avoid the danger of a precipitate forming on the smear by floating a cover glass on the stain or supporting a slide in a dish of stain with the smeared side down. With good

* Mallory, F. B., and Wright, J. H. Pathological Technic. Phila., 1924.

† Haden, R. L. *J. Lab. & Clin. Med.*, 1923, ix, 64.

stains and pure distilled water precipitation should not occur within the time of staining. Giemsa gives the same differential stain of blood elements as is obtained by the well-known Wright's or Leishman's solution. It is better for azure granules. Neutrophile granules are usually rather pale, especially if the smear is at all thick. It is an adaptable stain; variations can be made in the time of staining, in the dilution of the stain, and in the hydrogen ion concentration of the diluted stain.

b. After May-Grünwald Fixation. (1) May-Grünwald. This is a saturated solution in methyl alcohol of the eosinate of methylene blue. It contains the useful azure only as methylene blue contains some methylene azure. Used alone it affords a weak, unsatisfactory stain, but it improves the appearance of nuclei and is a more dependable fixation for Giemsa than alcohol. Jenner's stain is identical with May-Grünwald and may be used instead.

(2) May-Giemsa. The May-Giemsa or Jenner-Giemsa combination of Pappenheim is the popular stain for blood smears in most European laboratories. As with Wright's stain, the fixation is done with an alcoholic staining solution, but in this case the staining is completed with Giemsa as indicated below. The method can also be used for sections. Fix by covering the air-dried smear with May-Grünwald stain for three minutes. Add an equal amount of distilled water and leave the diluted stain for one minute. Pour off and without rinsing cover with diluted Giemsa solution (1 drop to 1 c.c. distilled water). Leave for fifteen to twenty minutes. Rinse off the stain and leave the smear in pure distilled water for about one minute or until differentiated. Blot dry and mount.

(c) After Acetone-lucidol Fixation. The acetone-lucidol method of St. Szécsi* also gives a good fixation of dry smears. Lucidol is a proprietary name for benzoyl peroxide, a white powder which explodes when heated to melting. Fix for fifteen minutes in a covered dish of benzoyl peroxide solution (10 gm. benzoyl peroxide, 100 c.c. acetone). Transfer without drying to a covered dish of acetone-xylol (acetone 3 c.c., xylol 2 c.c.) for ten minutes to dissolve the benzoyl peroxide crystals. Place in methyl alcohol for one-half to one minute. Stain in May-Giemsa, about five minutes in May-Grünwald diluted with distilled water, and about fifteen minutes in Giemsa. The method shows azure granules well.

4. Panchrome. *Pappenheim's panchrome* is a modified Giemsa stain. The powder obtained from the Grübler Company and made up according to their directions is brought into solution by adding 0.75 gm. to 75 gm. of methyl alcohol (pure and free from acetone) and 25 gm. of glycerin (acid-free) at 60°C. After being cooled it is filtered through a dry filter and kept well stoppered. It is used with May-Grünwald according to the directions given for Giemsa. The result is a better demonstration of neutrophile granules and better metachromasia of mast granules than the Giemsa

* Szécsi, S. *Deutsche med. Wchnschr.*, 1913, xxxix, 1584.

solution gives, but in our experience some delicacy of stain is lost, and the cells are more likely to be muddy.

5. The Kardos-Pappenheim Modification. This is also intended to make neutrophile granules more evident. Fix the smear with May-Grünwald for three minutes. Dilute with an equal volume of distilled water and leave for one minute. Pour off the solution and stain with Giemsa methyl green-orange mixture for fifteen minutes. Wash briefly in water, dry, and mount.

The Giemsa methyl green-orange stain is made, according to Kardos,* as follows: 2 per cent aqueous orange G solution is mixed with concentrated aqueous methyl green. The precipitate is dried and dissolved in methyl alcohol. The stain is made by taking 10 drops of Giemsa, 5 drops of methyl green-orange, and 15 c.c. of distilled water. It should be thoroughly shaken and poured from the mallow colored foam. Panchrome can also be modified in this way. The attempt is to combine the effect of triacid, the best stain for neutrophile granules, with that of May-Giemsa, the best stain for azure granules. Kardos states that with his modification a number of cells would be called neutrophilic promyelocytes, which with Giemsa alone would be called non-granular leucoblasts.

6. Triacid. Ehrlich's triacid is useful as a check on the Romanowsky stains because it colors neutrophile granules but not azure granules. It has been repeatedly stated by Ehrlich and Pappenheim that no conclusions should be drawn as to the presence or absence of neutrophile granules unless this test has been applied. Ehrlich used heat fixation of 110°C. for one-half to two minutes. A copper plate is heated by a bunsen burner at one end, and the air-dried smear is placed between the points where toluene (B.P. 110°C.) boils and water assumes the spheroidal state, 150°C. Other fixations are methyl alcohol twenty minutes or acetone five minutes. The triacid stain is used for five minutes. Then wash in distilled water until no more color comes away, dry and mount.

Triacid contains methyl green, orange G and acid fuchsin. A satisfactory stain is difficult to prepare, probably because methyl green is not a stable compound, either in a powdered form or in solution. It is a saving of time, therefore, to obtain it, as well as the above mentioned methyl green-orange solution, ready made from the Grübler Company.

C. WET SMEARS

I. Fixation

1. By Vapors. For amphibian blood in which the cells are large enough to be distorted by drying, a fixation of the moist film by vapor is to be recommended. Osmic acid 1 per cent should be used for twenty or at most thirty seconds by the following method. Support the clean slip or cover

* Kardos, E. *Folia haematol.*, Archiv. 1911, xii, 39.

glass over a dish of 1 per cent OsO_4 for a few seconds. Make the smear in the usual way on the surface that was exposed to the fumes, and put the moist preparation back over the fixative for twenty seconds. Then let it dry. Overfixation will make it stain too intensely. The appearance of nuclear structures is sometimes improved by the addition of glacial acetic acid to the osmic solution, 2 drops to 1 c.c. Excess acetic makes the blood colorless. Formalin vapor may be used instead of osmic for one to one and one-half minutes. Formalin and osmic acid are the most common reagents for this purpose, but Weidenreich* mentions others. In any case the preparation should be allowed to stand twenty-four hours before it is stained. Giemsa's stain is good after this fixation. If Wright's stain is used, the sixty second interval of concentrated stain should be omitted.

2. Agar-osmium Method of Deetjen-Weidenreich. An agar-osmium method was first used by Deetjen for blood platelets, but according to Weidenreich it is useful for all leucocytes. The writers have found it a valuable method for peritoneal fluid. It has the advantage that cells are fixed without being rolled between cover glasses or dragged over a slip. The following account is taken from Weidenreich:

Make a 1 per cent solution of agar in 0.8 per cent salt solution. Fill several test tubes with this to a height of about 1 inch, plug them with cotton, and sterilize them. To use, heat one of the tubes in hot water until the agar has become fluid and pour it out on a glass plate. After the agar has solidified, cut squares from it slightly smaller than the cover glass to be used. Place one of the agar squares on a slide. Then place a drop of peritoneal fluid or blood on a cover glass and lay the cover glass carefully on the agar square so that its edges project beyond the edges of the agar. The fluid spreads out in a thin layer between the glass and the agar. Leave the preparation for five or ten minutes and then add a few drops of 1 per cent osmic acid under the projecting edges of the cover glass but without touching the cover glass itself. After five minutes, carefully raise the cover glass and place it in distilled water for a few minutes. Stain in Giemsa without letting it dry. After staining, rinse the preparation in distilled water, dehydrate in 95 per cent alcohol and 100 per cent alcohol, clear in xylene and mount. Do not let it dry at any stage. This method is of value for mammalian material.

3. Zenker-formol Fixation of Bone Marrow, Lymph Nodes, and Spleen. Fixation of the wet smears directly in a liquid is used with such tissues as bone marrow or lymph nodes rather than with blood. For general preservation of leucocytes Maximow's modification of Helly's fluid is the best. Maximow uses for this fluid 100 c.c. of Zenker stock solution (without acetic acid) and 10 c.c. of neutralized commercial formalin. Magnesium carbonate may be used for neutralization. Fixation is unusually complete and granules are especially well preserved. It is used according to the following directions:

Float the cover glass on the fixative, smear side down, for fifteen minutes. Do not overfix. Wash in running water for two hours. Place in iodized 70 per cent alcohol for several hours or overnight. Remove excess iodine by treating with 0.25 per cent sodium thiosulphate solution for about five minutes and washing thoroughly in distilled water.

* Weidenreich, F. Die Leucocyten und verwandte Zellformen. Wiesbaden, 1911.

For best results stain within two days. The Helly-Maximow fluid gives especially favorable fixation for hematological stains because of the bichromate it contains.

II. Stains

Suitable stains are Maximow's azure 11-eosin, May-Giemsa, Giemsa, Dominici, indulin-aurantia-eosin and Ehrlich's triacid-toluidine blue. The use of these stains is described at the end of the section.

D. SPREADS

I. Preparation of Subcutaneous Tissue Spreads

Loose connective tissue is found in any vertebrate just under the skin, between that and the muscles. It is usually taken from the abdominal or inguinal regions. After the selected area is shaved, an incision is made in the skin and a very small piece of tissue is snipped off with scissors. The piece is placed on a cover glass and quickly spread out as flat and as thin as possible by means of two teasing needles, taking care, however, not to tear it to the extent of injuring the cells. It is an advantage for two people to work together in doing this, using two needles each. The cover glass is then floated on fixing fluid, the tissue side being down. All the above manipulations must be done quickly before the tissue dries, and it facilitates handling the cover glass if the teasing is done with the cover glass over a black background and supported by a Petri dish which is bottom side up.

II. Preparation of Omentum and Mesentery Spreads

There are several ways of taking omentum spreads: on filter paper rings, on test-tube tops, or the cover glass method used for subcutaneous tissue. The object is to get the membranes into the fixing fluid without tearing them or allowing them to dry, and to keep them perfectly flat and unwrinkled. If omentum is to be saved, it is the first thing taken out after the animal is killed. Care should be taken not to expose it to the air unnecessarily.

The filter paper ring method can be used by one operator working alone. A number of rings are cut of fairly heavy filter paper, about 5 mm. to 7 mm. in width and 1 inch in diameter. One of the rings is placed under the omentum so that the tissue is in contact with it all around. Immediately, the ring is cut out and placed in the fixing fluid. The tissue will stick to the filter paper through subsequent operations. After it is stained and taken into xylene, it may be cut from the paper ring as there is then no danger of its becoming wrinkled.

A method of spreading the omentum over the cut-off top of a test tube and tying it there with thread was used by Maximow. For rapid work this requires two operators.

In some animals, such as the frog, the mesentery is a hematopoietic organ. It is easily handled by leaving it attached to the loops of intestine and placing the whole in fixing fluid.

III. Fixation and Stains for Connective Tissue Spreads

For all connective tissue spreads the best general fixation is Zenker-formol used just as described for smears (p. 249). If mast cell granules are to be preserved, the use of watery fixatives must be avoided and the tissue placed directly in absolute alcohol. Alcohol serves as a quick fixative requiring no washing out, but it makes many cells look vacuolated because of its dissolving action on certain cell constituents. Formalin, also, gives a quick fixation, and for general purposes it can be sufficiently washed out in a few minutes so that the tissue will take a stain. Preparations of subcutaneous tissue and omentum should be stained within a few days after fixation. However, if they have been left for a week or so in alcohol so that some staining capacity is lost, the fixation can be revived by the use of peroxides. The same stains are valuable here as are used on sections.

E. SECTIONS, PARAFFIN OR CELLOIDIN

In a study of leucocytes the paraffin method is useful for quick tests and for most ordinary purposes. The celloidin method is more valuable, however, as there is less shrinkage, and the delicate structures of the cells are better preserved. In cases where difficult material must be cut, it is the only practicable method. The celloidin must be removed in order to treat sections with hematological stains.*

I. Fixation

The usual fixatives may be used, but the time is necessarily longer than for smears and membranes. Zenker-formol, the best fixative, is used for five hours if the piece is of ordinary size, 1 cm. × 1 cm. × ½ cm. Very small pieces are penetrated in three hours. This is followed by twenty-four hours' washing in running water. Formalin is used for twenty-four hours in the strength of 10 c.c. commercial formalin and 90 c.c. of water. After twenty-four hours this should be diluted with an equal volume of water if pieces are to be left in it for a longer time. Bell has found that better results are obtained with old formalin material if dehydration is carried out very slowly, twelve to twenty-four hours in each grade of alcohol. A mixture of corrosive sublimate and formalin preserves megacaryocytes well. Its use is described in connection with megacaryocytes (p. 255). Absolute alcohol is used for pieces of tissue only when watery fluids must be avoided, as in the preservation of mast cell granules.

* Dantschakoff, Wera. *Ztschr. f. wissensch. Mikr.*, 1908, xxv, 32.

II. A Method for the Preparation of Body Wall

The following method for sections of body wall is taken from Maximow,* 1906.

Shave the skin, make a cut through the body wall and insert a cork frame. Fasten the whole thickness of the body wall to the cork by means of pins if alcohol is used as the fixative, or thorns for a corrosive fluid. Then cut out the cork frame with the tissue pinned to it and carry it through the operations of fixing and hardening. In 80 per cent or 90 per cent alcohol remove the tissue from the cork and trim its edges. Imbed in celloidin, and, in order to get the cells of the subcutaneous connective tissue, section the piece parallel to the surface. This method is suitable for the smaller animals but not for dogs because in them the body wall is too thick. Instead, layers of muscle may be pinned out with connective tissue between them. In fixing body wall in absolute alcohol, Unna states that tannin should be avoided and corks should be soaked in sodium carbonate solution of 2 per cent before use.

F. STAINING PREPARATIONS FOR LEUCOCYTES

1. Delafield's hematoxylin and eosin, the best known tissue stain, shows up general features well in sections of glands or organs especially of human material, but is otherwise little used in a study of leucocytes.

2. Azure II-eosin and hematoxylin as used by Maximow,† 1909 and 1924, is a valuable combination which is generally applicable; it can be used on Zenker-formol fixed sections, smears, or spreads, and also on tissue culture preparations. Sections of paraffin or preferably celloidin material are stained first in Delafield's hematoxylin and afterwards in azure II-eosin. The Delafield's stain, not artificially ripened, should be about six weeks old. Make a very dilute solution of it by adding 1 to 2 drops to 100 c.c. of redistilled water. It is as necessary to use pure distilled water here as in working with Wright's blood stain. The solution will be a pale violet color if the Delafield's and the water are both good. Leave sections in the stain for twenty-four hours. Then wash in distilled water for about twenty-four hours. As a result chromatin is blue, and the cytoplasm is either colorless or a light grey. Place in azure II-eosin solution for twelve to twenty-four hours.

Azure II-eosin solution:

Stock solution A.

Eosin, water soluble yellowish (W.G.)	0.5 gm.
Pure distilled water	500.0 c.c.

Stock solution B

Azure II	0.5 gm.
Pure distilled water	500.0 c.c.

Stain

Solution A	10 c.c. or 12 c.c. according to material
Solution B	10 c.c. or 9 c.c. according to material
Distilled water	100 c.c.

* Maximow, A. *Arch. f. mikr. Anat.*, 1906, lxvii, 680.

† Maximow, A. *Ztschr. f. wissench. Mikr.*, 1909, xxvi, 177
Maximow, A. *J. Infect. Dis.*, 1924, xxxiv, 549.

Mix the eosin solution and the water first, and then add the azure solution. A noticeable precipitate should not form for several hours. Leave slides upright in the stain for twenty-four hours. Transfer from the stain to 95 per cent or 100 per cent alcohol to differentiate and dehydrate. Finish the dehydration in a second 100 per cent alcohol. Clear in xylene and examine under a microscope. The slides can be further differentiated in 100 per cent alcohol if necessary. Mount in pure, petrol ether-extracted damar, evaporated to dryness and redissolved in xylene C.P.

By this staining combination the chromatin of the nuclei is dark blue, nucleoli are purple and leucocyte granules are differentially stained in colors corresponding to those given by Giemsa. Mast granules may be dissolved away by prolonged treatment in watery fluids. The azure II-eosin solution may be used without hematoxylin. Also, Delafield's hematoxylin may be used in this way preceding such stains as Dominici and Giemsa.*

3. Von Möllendorff's† iron hematoxylin method is intended to demonstrate connective tissue cells of the subcutaneous layer, showing even their finest processes. According to its originator, it is difficult to use. A hematoxylin solution is made by dissolving 1 gm. of dye in 10 c.c. of absolute alcohol, diluting with distilled water to 100 c.c., and letting it ripen for two or three weeks. After four or five months it becomes useless. Just before use, this solution is mixed with 2 per cent iron alum. As the proportions depend on the stage of ripening of the hematoxylin, each mixture must be tried out (Iron alum: Htx = 1:1 — 3:1). At 56°C. the mixture should precipitate only after five or ten minutes. It is at first violet, but after thirty seconds becomes blue. An immediate precipitate means too much hematoxylin. If there is no precipitate after ten minutes and the solution has a green-brown color, too much alum was added proportionally. The staining mixture can be used only once. Stain for ten minutes at 56°C. and differentiate with 2 per cent iron alum, controlling for the best result. The cytoplasm of fibroblasts should be fully stained, while, if the method is successful, the collagenous fibrils are colorless.

May-Giemsa and the Dominici stain described below give a differential coloration of blood elements and are useful for smears, spreads, or sections. They act more quickly than Maximow's azure II-eosin, but they are so sensitive to minute details of technique that it is almost impossible to get a series of preparations stained in a uniform manner.

4. May-Giemsa is used in a way very similar to that described for smears: May-Grünwald (diluted) five minutes, Giemsa (1 drop to 1 c.c. distilled water) fifteen minutes. Then wash the section in water, dehydrate in 95 per cent and 100 per cent alcohol and clear in xylene. The stain can be regulated by varying the pH of the diluted solution. If the section has taken too blue a tone, the eosin color can be brought out by washing in very dilute acetic acid before dehydrating. Another useful variation is the

* We wish to express an obligation to Dr. Maximow for checking this description.

† v. Möllendorff, W. and Milie. *Ztsch. f. Zellforsch. u. Mikr. Anat.*, 1926, iii, 503.

use of acetone for dehydration and cedar oil followed by xylene for clearing. May-Giemsa is a satisfactory stain only on material which is very well fixed.

Panchrome and the Kardos modification of panchrome or Giemsa are said to be excellent stains for sections.

5. **Dominici** requires the use of two solutions. First, stain for six or seven minutes in an eosin-orange G solution (eosin ½ gm., orange G ½ gm., and distilled water 100 c.c.). Rinse off the excess stain in distilled water but do not leave the section in water long enough to fade. Then stain in 0.5 per cent aqueous toluidine blue solution for twenty to thirty seconds. Rinse in distilled water and differentiate in 95 per cent alcohol, controlling the result by a rapid glance at the preparation under a microscope. Finish the dehydration with 100 per cent alcohol and clear in xylene.

6. **Methyl green-pyronin** (Pappenheim) is particularly brilliant on sections of spleen and lymph nodes because it gives a bright red color to the cytoplasm of large lymphocytes and plasma cells. It is especially valuable when it is desired to distinguish between chromatin and other basophilic materials in the cell, as the method is specific for chromatin. The latter is stained a dark, greenish-violet color, while other basophilic constituents of the cell are colored red with the pyronin of the mixture. The nucleolus is red if of basic staining reaction and colorless if acid. The method is, therefore, a very convenient one for distinguishing between chromatin and nucleolar material. It should be used after Zenker-formol or alcohol fixation. It cannot be used after formalin alone. The formula follows:

Solution A	
Phenol crystals, liquified	0.25 gm.
Distilled water	100.00 gm.
Methyl green	1.00 gm.
Solution B	
Phenol crystals, liquified	0.25 gm.
Distilled water	100.00 gm.
Pyronin	1.00 gm.
Stain	
Solution A	15 parts
Solution B	35 parts

After several weeks or months a precipitate may form in the staining solution. Stain for about six minutes. Rinse briefly in distilled water, dehydrate in acetone, and clear in cedar oil followed by xylene.

7. **Alcoholic thionin** (50 per cent alcohol saturated with thionin) is used on material which has been fixed in absolute alcohol for the preservation of mast cells. Stain for twenty-four to forty-eight hours in a covered dish. A solution which acts more quickly can be made by adding 4 drops of 2 per cent Na_2CO_3 to 20 c.c. of the thionin solution and leaving it overnight. This solution should be filtered before use. Stain for ten to twenty

minutes. Thionin is a metachromatic stain and colors nuclei blue and mast cell granules a brownish-red to violet or purple color.

8. Indulin-aurantia-eosin (Ehrlich) and Ehrlich's triacid-toluidine blue have their greatest usefulness on marrow smears and similar material fixed in Zenker-formol.

The indulin-aurantia-eosin formula is as follows:

Indulin	2 gm.
Aurantia	2 gm.
Eosin	2 gm.
Glycerin, pure	30 gm.

Dissolve the mixture in the oven at about 40°C. Keep the stain well stoppered. To use, cover a smear with stain and place it in an oven at 40°C. for four to five and one-half hours. Rinse in distilled water. Dehydrate in 95 per cent and 100 per cent alcohol. Clear in xylene. This stain gives an excellent differential result on granules. Preparations keep well.

9. Ehrlich's triacid-toluidine blue is one of the most brilliant stains for bone marrow cells. According to our experience, it fades in a year or two. The triacid stain is discussed in connection with dry smears. For smears fixed in Zenker-formol the following procedure is used: Place the smear in triacid for five minutes, rinse briefly in distilled water and stain in 0.5 per cent aqueous toluidine blue for ten seconds. Then rinse again in water, dehydrate beginning with 95 per cent alcohol and clear in xylene.

Megacaryocytes and Blood Platelets

Methods for counting blood platelets are described by Isaacs (p. 221).

For a histological examination the Romanowsky stains are good. Smears treated with Wright's blood stain, Giemsa, or the modifications of Giemsa show a sharp differentiation of the characteristic azurophil granules in platelets and megacaryocytes, no matter whether they are used on the ordinary dry smears or on those fixed as quickly as possible while still moist.

In sectioned material, Schridde was the first to demonstrate the granules of megacaryocyte cytoplasm using azure II-eosin followed by dehydration in acetone. Shortly after this, Wright worked out a modified Romanowsky combination which is known as Wright's megacaryocyte stain.* Wright's method follows:

Fix in a saturated solution of mercuric chloride in 0.9 per cent salt solution. Dehydrate in alcohol followed by acetone, clear in thick cedar oil followed by xylene, and imbed in paraffin.

Stain sections for ten minutes in a mixture of equal parts of staining fluid and distilled water, poured directly on the slide. A yellowish metallic scum should slowly form, but the stain should not precipitate. The time may be varied. When the cytoplasm of the giant cells is bright red and reticular fibrils begin to look red, the staining should be stop-

* Wright, J. H. *J. Morphol.*, 1910, xxi, 263.

ped, and the preparation should be at once washed in water. Dehydrate in pure acetone and clear in pure oil of turpentine. Mount in a thick solution of colophonium in pure oil of turpentine. Before mounting carefully remove superfluous turpentine as it may absorb moisture and cause clouding or fading. Downey* found that turpentine-damar works equally well.

Make the stain by taking 3 parts of polychrome methylene blue solution and 10 parts of 0.2 per cent solution of eosin water soluble yellowish (W.G.) in methyl alcohol. For the polychrome methylene blue solution dissolve 1gm. of methylene blue B.X. in 100 c.c. of a 0.5 per cent aqueous solution of $NaHCO_3$ and keep the solution at 100°C. in a steam sterilizer for one and one-half hours. Filter the solution when it is cool. The filtrate is polychrome methylene blue.

Preparations stained in this way show both platelets and megacaryocytes in good detail. Granules are clearer if they are examined by light from an electric bulb which has a yellowish tint. In order to secure satisfactory results, it is absolutely necessary to follow Wright's technique in detail, including the use of acetone and turpentine.

Downey found that the corrosive sublimate fixation of Wright's method, although excellent for megacaryocytes and platelets, was poor for lymphocytes in the same preparation. He substituted a mixture of 10 c.c. commercial formalin and 90 c.c. of 0.9 per cent salt solution, saturated with $HgCl_2$. The solution gives a good fixation of lymphocytes as well as megacaryocytes, and allows not only a typical Wright's stain but also a fairly good stain with the Giemsa and Dominici mixtures.

* Downey, H. *Folia haematol.*, Archiv., 1913, xv, 25.

HISTOLOGICAL TECHNIQUE FOR THE STUDY OF BONE

P. G. Shipley

General considerations 257. Preparation of sections of whole bone without decalcification 258. Preparation of bone from which organic matter has been removed 258. Decalcification 259. Isolation of bone corpuscles 261. Isolation of lamellae 261. Staining 261. Demonstration of calcified tissue in bone 261. Demonstration of lacunae and canaliculi 262. Demonstration of processes of young osteoblasts in growing area of bones 263.

I. GENERAL CONSIDERATIONS

Histological methods which have been devised especially for the study of bone, in general have to do with the removal from this tissue of the lime salts with which it is impregnated, and which, unless they are removed, make it impossible to cut the bone into sections for study. These methods depend for their efficiency on the solubility of the tertiary phosphate and the carbonate of calcium in strongly acid solutions and are all equally efficacious, and satisfactory in that they remove the inorganic material from the bones and leave the tissue softened and in a proper state for cutting. The majority, however, fail because the strongly acid solutions so sear the soft tissues to the calcified structures, and cause so much destruction and shrinkage as to make the normal structure and relations of the cellular tissue highly conjectural. In order to obviate this difficulty many authors have suggested the use of weak organic acids. While decalcification with the latter allows the preparation of much better sections, both of the calcified and soft tissues, it is extremely time consuming, and requires a great deal of patience in application. The good results obtained by the use of these weak decalcifying agents, nevertheless, are worth the time and patience required.

It must not be forgotten in this connection that certain reagents used for fixation or hardening soft tissue, will, if their action is long enough continued, decalcify even quite large calcareous bodies. This action is dependent on the presence or evolution in them of small amounts of one acid or another, and may be abolished by neutralization.

The *calcified part* of bone is studied by methods which have been devised for making it visible after destruction of the organic adnexa. Moreover, certain special ways have been worked out for the easy demonstration of some of the structures peculiar to osseous tissue.

Bits of bone which are sufficiently small and included in soft tissue may be cut after being imbedded in celloidin but they will invariably destroy the edge of the knife, which will require constant sharpening.

II. PREPARATION OF SECTIONS OF WHOLE BONE WITHOUT DECALCIFICATION

The following method was worked out by Grieves for the study of dental tissues and is eminently satisfactory for preparing sections of bone with the soft tissues attached:

1. Small bones or pieces of bone with marrow or other soft tissues are fixed for twenty-four to thirty-six hours in formalin 10 per cent or other desired fixing agent.
2. Wash in running water twenty-four hours, then one hour each in 2 changes of distilled water.
3. Dehydrate in graded alcohols beginning at 50 per cent.
4. Treat with absolute alcohol and chloroform, equal parts, two hours.
5. Follow with chloroform two hours.
6. Pass the tissue into solutions of window glass rosin* in chloroform as follows: Two hours each in 5 per cent and 10 per cent solution, then into a saturated solution until it becomes transparent. Finally the tissue is imbedded in rosin in the following manner:

Three small glass dishes containing melted rosin are maintained in that condition on a copper bar, and the bone is passed through them, remaining one minute in each. Evaporation of any chloroform remaining from the final solution of rosin in chloroform occurs during this process. The block is then allowed to cool and is ground as thin as possible by hand on carborundum stones. One surface having been polished on a fine hone, it is fastened to a warm glass slip with a bit of melted rosin, care being taken to exclude air bubbles between the section and the slip. It is then rubbed down to the desired thinness. The remaining surface having been polished on a very fine hone or on glass, the section is ready to be treated by the method of staining described below. All grinding must be done under lukewarm water.

III. PREPARATIONS OF BONE FROM WHICH THE ORGANIC MATTER HAS BEEN REMOVED

These are made by digestion in alkalies, or by tryptic digestion, by calcination, by drying in air or in sand, through the agency of insects as by exposing the tissue to ants, by bacterial action involving putrefaction and by autolysis. Perhaps the best method for accomplishing this result is to trim and scrape all the soft tissue away from the bone which may or may not be split. The bone is then placed in tap water or in a weak (2 per cent) solution of gelatin to which a loopful of a culture of B. coli has been added. In about five or six days the bone is removed from the culture and thoroughly rinsed in running water for twenty-four to forty-eight hours. All the organic matter will, as the result of this procedure, have been dissolved and washed away. The whole process should of course be carried out in an unfrequented and well-ventilated place as the odors evolved during putrefaction are intolerable. It is as well to sterilize bones so prepared, by boiling, or by immersion in alcohol before going further with their treatment. When the bone has dried completely it may be sawn into sections,

* Window glass or water white "rosin" (not resin) is a very pure transparent pine rosin used in dental technique and may be obtained from dealers in dental supplies.

which may be ground to the requisite degree of thinness either by hand or on such an apparatus as Coriel's laps or on Black's instrument. The finished section should be polished on glass or a fine hone and after dehydration in ether should be mounted, when thoroughly dry, in thick balsam and covered.

IV. DECALCIFICATION

1. With Solutions of Inorganic Acids. Hydrochloric acid has long been used for decalcification in 1 to 2 per cent and 3 per cent watery and alcoholic solutions and in 5 per cent solution in glycerin. Its action is very rapid but it is not to be recommended as it swells the tissues greatly and I do not feel that such substances as salt, chromic acid or alcohol have any effect in preventing the results of its deficiencies. I have had no experience with mixtures with palladium chloride.

a. Nitric Acid. Like hydrochloric acid, nitric acid can only be recommended for use when very large masses of bone are to be decalcified very rapidly. It may be used in strengths varying from 1 to 10 per cent in either alcoholic (70 per cent or 95 per cent) or watery solution which should be changed every day. After decalcification the tissue should be washed in running water and transferred to 95 per cent alcohol, which should be changed three times. It is better to bring the tissue into 95 per cent alcohol before putting it into the decalcifying fluid.

b. Sulphuric acid is perhaps the poorest decalcifying agent.

c. Sulphurous acid is used in saturated solution after fixation in formalin. It is said to preserve tissue well.

d. Formic acid for softening large masses of bone is to be preferred to any other. It may be used in watery solution but is best at a 1 to 5 per cent strength in 70 per cent alcohol. Tissues should be fixed in formalin and after decalcification must be washed in 70 per cent alcohol, not water. Decalcification in 5 per cent solution goes to completion in four to five days even when large masses of bone are used.

2. With Weak Acids. After extensive experimentation with many methods of decalcifying bone the writer has come to rely on prolonged immersion in Müller's fluid as the best and most reliable means. The decalcification is effected through the liberation of small amounts of chromic acid from the potassium bichromate in the solution. Bones are sawn or cut into pieces or split and allowed to remain in formalin (10 per cent solution) for seventy-two hours and then immersed in Müller's fluid and kept in it until they bend or can be pierced easily with a needle. The fluid should be changed occasionally but not too frequently, not oftener than once in two weeks, since more frequent changing slows the decalcification very much. Decalcification by this method is very slow but the results obtained through its use fully compensate for the delay. Removal of the lime from the bones of a normal adult rat occupies from twenty-one to thirty days in Müller's fluid. The process may be hastened somewhat by being carried out in an incubator at 37°c. Over-decalcification is practically impossible in this fluid. This is the only decalcifying agent which allwso

the determination of the amount of osteoid tissue present in the bone during life, since all lamellar substance which was calcified is basophilic and stains a more or less intense blue with hematoxylin. Osteoid tissue alone is acidophilic. Soft tissues are beautifully preserved. This fluid may be followed by almost any stain including those used for coloring mitochondria.

a. Chromic acid is often recommended alone and in mixtures with other acids as a decalcifying agent, e. g., equal parts of 3 per cent chromic acid and 1 per cent hydrochloric acid. Solutions containing chromic acid alone are very weak agents and cause much shrinkage. One-half per cent is the best solution to use. Mixed with other acids chromic acid fails to prevent undesirable effects. The following acids are used as indicated:

b. Picric acid in saturated solution is very weak but can be used for small objects.

c. Phosphoric acid is used in 10 per cent to 15 per cent solution. This acid ruins staining possibilities in most tissues. For very small bones it may be taken in solutions of 0.5 per cent.

d. Lactic acid in solutions of 10 per cent strength is recommended by some workers. This acid causes much swelling.

e. Arsenic acid. 5 per cent is the strength recommended in which this acid should be used. I have no experience with results from decalcification by this substance.

f. Trichloractic acid decalcifies in 4 to 5 per cent solution.

3. With Phloroglucin.

Phloroglucin is always used in combination with acids such as hydrochloric, nitric, or sulphuric. It is not a decalcifying reagent but protects soft tissues against the strong acids and allows their use in high concentration. A saturated solution of phloroglucin is made, and to it are added 5 to 40 per cent of nitric acid. After decalcification the bone may be washed in running water. Another good formula is the following:

Phloroglucin	1.0 gm.
Nitric acid	5.0 c.c.
Alcohol	70.0 c.c.
Distilled water	30.0 c.c.

The latter decalcifies somewhat more slowly.

4. With Magnesium Citrate.

Kramer and the author have evolved a method of decalcification in neutral solutions which is very satisfactory for small pieces of material which have been fixed in formalin. This method depends on the solubility of tertiary calcium phosphate in water in the presence of citrate of magnesia. The reagent is prepared as follows: 80 gm. of citric acid are dissolved in 100 c.c. of hot water; 4 gm. of magnesium oxide are added and the mixture is stirred until a complete solution is made. Cool and add 100 c.c. of ammonium hydroxide (density 0.90) and dilute to 300 c.c. Allow to stand twenty-four hours and filter. (If the magnesium oxide contains much carbonate it should be freshly ignited.) Titrate with 5^{N}/hydrochloric acid to approximately pH 7.0 — 7.6 and add an equal volume of distilled water. This solution should be changed every three days. It decalcifies with moderate rapidity (fifteen days for a dog's rib) and preserves the architecture of soft tissue very well. Best results are obtained by using large quantities of the solution. Ammonium citrate has been used for the same purpose.

V. ISOLATION OF BONE CORPUSCLES

This is easily carried out as follows: A thin section of bone is put into concentrated nitric acid for from a few hours to a day. The section is then placed on a slip with a cover glass over it. Pressure on the cover will squeeze out the elipsoid bone cells with their processes.

VI. ISOLATION OF LAMELLAE

This may be accomplished by decalcifying bones and then allowing them to simmer in water. The lamellae will peel off readily after this treatment, often by themselves.

VII. STAINING

To prepare bone, there would seem to me to be no better fixative than a 10 per cent solution of formalin. Almost any method which can be applied to any other material may be used to color sections of osseous tissue. For ordinary purposes the writer knows of none which give better results than hematoxylin and eosin, although some prefer carmine as a contrast stain. Iron hematoxylin and phosphomolybdic hematoxylin give very beautiful preparations. Celloidin is much to be preferred to paraffin as an imbedding matrix.

VIII. DEMONSTRATION OF CALCIFIED TISSUE IN BONE

To demonstrate the calcified tissue in bone, special stains for osseous tissue are used. The lamellar tissue which was impregnated with lime during life is basophilic after decalcification in Müller's fluid or magnesium citrate and is colored blue by hematoxylin, blue-black by phosphomolybdic and black by iron hematoxylin, etc., but the most convenient method of demonstrating the extent of calcification is that of v. Kossa. This depends on the formation and reduction of silver phosphate or carbonate and the precipitation of metallic silver under the influence of light. It may be carried out with frozen or celloidin sections or with the surfaces of blocks of fresh bone. Material as described above is immersed in a 1 per cent solution of silver nitrate, after washing in distilled water to clean the tissue of blood or debris. It is then exposed to the full light of an arc or tungsten filament in the nitrate solution and allowed to remain until no further blackening occurs. It should be removed before the soft tissues begin to turn brown. This staining does not penetrate deeply into the bone. The tissues must not have been exposed to acids before immersion lest some of the lime salts be removed, but they may be decalcified after the precipitation of the silver since the latter metal remains in situ in spite of the removal of the calcium. Preparations made by v. Kossa's method are not permanent, but they may be kept a few months if they are well washed in distilled water and treated with sodium thiosulphite to remove the excess of silver salts after impregnation.

IX. DEMONSTRATION OF THE LACUNAE AND CANALICULI

The simplest method of demonstrating the lacunae and the canaliculi of dried bone is as follows: 1. Sections of bone ground to required thinness are placed in 0.75 per cent silver nitrate solution and allowed to remain twenty-four hours. After washing the section, polish it on a fine hone to remove the precipitated silver, dehydrate in alcohol and imbed in balsam from xylol. Lacunae and canaliculi are black or yellowish brown.

1. Impregnation with Acid Fuchsin. Thin sections of dried bone are extracted with alcohol and dried thoroughly. They are then put in watch crystals containing a 20 per cent solution of acid fuchsin. The dishes are placed in a desiccator which can be connected to a suction apparatus. The air is extracted for about an hour and the desiccator locked. After twenty-four hours the solution will have dried. The sections should be removed and the superfluous precipitate rubbed off on a fine carborundum stone. They should then be polished on a hone and passed through xylol to be mounted in damar or balsam.

2. Methods for Staining Linings of Lacunae and Canaliculi. The linings of the lacunae and canaliculi can also be stained beautifully by the following:

a. Krause's Method for Frozen Sections.

(1) Gram's solution, 25 c.c. Water, 25 c.c., one-half hour.
(2) Wash.
(3) Gold chloride 2 per cent solution until the sections are yellow.
(4) Wash and reduce in a 2 per cent solution of resorcin, one to two hours.
(5) Wash.
(6) 5 per cent solution of hyposulphite of soda, fifteen to thirty minutes.
This method is recommended by Krause for frozen sections.

Of the two staining methods described by Schmorl the writer has found the second by far the more satisfactory. The methods are given as the writer has used them:

b. Sections in Celloidin (Modified Schmorl Methods).

First Method.

(1) Fix in formalin.
(2) Decalcify in Müller's fluid.
(3) Wash, dehydrate and imbed in celloidin.
(4) Wash in water thirty minutes.
(5) Stain in the following solution for ten minutes:

Saturated solution of thionin in 50 per cent alcohol	2 c.c.
Water	10 c.c.

(6) Wash.
(7) Decolorize (one half to one minute in a saturated solution of picric acid).
(8) Wash.
(9) Treat with 70 per cent alcohol until the color ceases to come off rapidly.
(10) Dehydrate in 95 per cent alcohol.

(11) Clear in oil of origanum.
(12) Mount in damar.

Second Method.

(1) Fix in any fluid not containing mercury.
(2) Decalcify in Müller's fluid.
(3) Wash in running water.
(4) Dehydrate, imbed in celloidin, cut sections not over 10 μ.
(5) Stain in thionin solution made alkaline with 2 drops of ammonia.
(6) Transfer with a glass needle to a saturated solution of phosphotungstic or phosphomolybdic acid until the sections are blue or gray or green.
(7) Place in water until sections are sky-blue.
(8) Transfer into the following solution for three to five minutes:

Ammonium hydroxide	1.0 c.c.
Water	10.0 c.c.

(9) Pass through several changes of 90 per cent alcohol and into
(10) 95 per cent alcohol.
(11) Clear in carbol xylol.
(12) Mount in damar.
This method is recommended for the bones of children.

X. DEMONSTRATION OF THE PROCESSES OF YOUNG OSTEOBLASTS IN THE GROWING AREA OF BONES

Thin slices of bone from a rickety animal are cut and treated in the manner following:

(1) Place in a 4 per cent solution of citric acid in distilled water for twenty to thirty minutes in the dark.
(2) Rinse in distilled water.
(3) Transfer to a 1 per cent solution of gold chloride in distilled water in the dark for twenty to thirty minutes.
(4) Place in a 33 per cent solution of formic acid in the dark for forty-eight hours.
(5) Rinse in distilled water.
(6) Preserve in pure glycerin.

Frozen sections should be made and mounted in glycerin. Ring the cover with damar, balsam, paraffin or cement. When not in use the slides should be kept in the dark.

1. Grieves' Stain for Sections of Hard and Soft Tissues. The slide prepared as described above is rinsed in distilled water and treated as follows:

(1) 20 per cent alcohol fifteen minutes
(2) Stained in cresylechtviolet solution twelve to twenty-four hours according to the thickness of the section.

(a) Stock solution of cresylechtviolet	1 part
(b) Distilled water	99 parts

The stock solution of the dyestuff is made as follows:

Cresylechtviolet	1 gm.
Phenol (5 per cent solution)	50 c.c.
Alcohol 95 per cent	20 c.c.

The dye should be thoroughly suspended in the phenol before the alcohol is added.

(3) Rinse in distilled water.

(4) Differentiate in acetone.

(5) Clear in bergamot oil and creosote.

(6) Mount in damar.

The acetone differentiates the stain, dehydrates the section and dissolves the rosin with which it was impregnated.

DENTAL HISTOLOGICAL TECHNIQUE

J. L. T. Appleton, Jr.

Examination of tooth as a whole 265. Examination of special structures in dental histology 271.

By the "dental tissues" we mean: 1, the enamel cuticle or Nasmyth's membrane; 2, the enamel; 3, the dentin; 4, the cementum; 5, the pulp; 6, the alveolo-dental periosteum or periodontal ligament and 7, the alveolar bone. The chemical composition, the physical nature and the structural arrangements of these parts offer difficulties in their histological examination which have been met in various ways.

Many structural details of enamel, dentin, cementum and alveolar bone can be made out in sections prepared by grinding. To see the dental tissues in their relation to each other it is necessary to use first, some decalcifying method followed by microtome sectioning, and secondly, some method of petrifaction followed by grinding.

A. EXAMINATION OF THE TOOTH AS A WHOLE

1. Simple Grinding. If it be desired to preserve the enamel, the specimen should at no time be allowed to become dry. Drop the tooth into a physiologic sodium chloride solution with 4 per cent formalin, immediately after removal from the body. If the grinding is to be done within a few hours, the physiologic solution alone will do. Divide the tooth in the plane of the section by cutting it with a fine saw while it is securely held in a vise between two pieces of cork.

The grinding can be done in three ways: 1, by hand on a flat carborundum stone; 2, on a carborundum wheel driven by a motor; or 3, between two plates of frosted glass. The combination carborundum stone No. 108 (5 cm. × 120.4 cm.) is very satisfactory for hand grinding. On one side is grit No. 120, on the other 2 f. The coarser grit should be used only long enough to get the two sides of the tooth parallel, then the grinding should be continued on the finer side. The surface should be constantly flooded with water. A good way is to lay the stone in a glass photographic developing tray of sufficient size, with a blotter on the bottom, to keep the stone from slipping. Water can be kept in the tray almost to the level of the upper surface of the stone. From this as a reservoir the surface can be frequently flooded. When the section has almost reached the desired thickness, it should be transferred to a very fine stone (carborundum, "60 minute" grit or an Arkansas stone) for final polishing. Polishing can also be done on plate glass with a thin paste of the finest precipitated chalk. The specimen, while it is being moved back and forth, is conveniently held by a cork, in size large enough to cover the specimen and about 10 to 15 mm. high,

which permits the fingers easily to take hold. The grinding should never be so fast as to cause cracking. The optimum rate is learned only by practice. If one side of the tooth is being ground down more quickly than the other, this can be corrected by altering the direction of pressure on the cork.

When the polishing is completed, transfer the section by means of a camel's hair brush and section lifter to a dish of clean distilled water, and there thoroughly free it from the debris of grinding. Several changes of water are necessary. Then pass the section through the alcohols, into xylol and mount in balsam. It is often well to hold the cover glass down gently with a wire spring until the balsam has hardened.

The interglobular spaces in the dentin and the branchings of the dentinal tubules will be rendered more conspicuous if they contain air before the balsam is added. Consequently for this purpose remove the section, after immersion in 95 per cent alcohol, to the slip, and blot firmly against it. If the air of the laboratory be dry, exposure for an hour or two, before putting on the balsam and cover glass, will suffice. In this case the wire spring to hold the cover glass in place will be almost indispensable, because on drying, the section, if very thin, usually becomes warped, which is highly undesirable when it comes to microscopic examination. It should be remembered that cracks usually appear, particularly in the enamel, when the section is allowed to become dry.

2. Grinding against a Revolving Wheel-stone. The grinding can be greatly accelerated by the use of motor-driven, wheel-shaped stones with a coarse and a medium grit wheel. The position of the stone may be either vertical or horizontal. The grinding is done against the flat surface of the wheel, which should, before use, be "trued" by holding against it while rapidly rotating a "trued" flat carborundum stone of the same grit. A constant flow of water should be kept over the surfaces in contact. The stone can be kept moist at this time and for the grinding of the tooth by means of a simple system, consisting of a small, fine-pored sponge lightly held in contact with the revolving stone and supplied with water through a small-bored tube. The flow of water can be regulated by means of a screw pinch-cock when the connecting tube is of rubber. The "trueing" of the carborundum wheel need be done only when first purchased.

The coarser stone should be used only until the surfaces of the tooth are parallel. Most of the grinding will be done with the medium stone. At first it will be easier to hold the specimen with the fingers but when the surfaces are once parallel, holding by means of the cork can be substituted. During the grinding the tooth should be constantly moved back and forth along the line of a radius of the stone. The speed of rotation should not be great and the specimen should not be pressed tightly against the cutting surface. It is of advantage to be able to increase the speed of the motor gradually.

The final polishing must be done by hand on a very fine stone.

Sections of the hard dental tissues may be ground between two plates of ground or frosted glass. The larger, *ca.* 8 inches square, is placed, smooth side down, on a wet towel or a wet piece of newspaper, one layer thick, to keep it from sliding. On the upper surface is placed a pinch of finely powdered glass or pumice stone or carborundum powder with plenty of water, and one surface of the specimen is ground flat. The specimen is held in the fingers and a broad, rotary motion is used. When one surface has been ground flat, the specimen is turned over and the other surface is flattened. The specimen must be held in the fingers until the section is half a millimeter or less thick. Then the upper square of frosted glass (*ca.* 4 inches square), smooth side up, is put over the specimen and water run in from the side so that almost the whole space between the two pieces of glass is filled with water. The abradant should be used sparingly. The upper plate, held with the fingers, is then moved with a broad, not too rapid rotary motion. The specimen is easily seen through the upper piece of glass. Grinding is continued until the section almost falls apart. When this is likely to happen can only be learned by experience. Considerable practice should be acquired, in this method as well as in any other grinding method, before one begins to work with important or valuable specimens. The section is removed carefully for final polishing on a very fine stone, by hand.

Hand grinding is tedious and grinding against a motor-driven wheel as just described is almost as bad. Consequently several machines have been devised to make the operation as automatic as possible.*

The principle underlying the construction of these machines is the same. The motor-driven grinding stone is fixed in a vertical position at one end of a lathe. One side of the specimen is ground flat and polished. This side is cemented by balsam to a brass section-holder. Several sections may be so cemented at one time. The "proper" consistency of the balsam is of the utmost importance. It should be just thick enough to become solid, but not brittle, in ice water. If the balsam is too thin, the slice will "creep" on the surface of the object disc during grinding. If it is too thick it becomes brittle and the slice breaks from the object disc. The most desirable consistency seems to vary considerably in different lots of balsam and can be determined only through personal experience. Black states that it should be stiff enough to move sluggishly at 110°F., but fluid at 120°F. or 130°F.†

This carrier is affixed to the end of a horizontal shaft, held without play in a casing which slides on the lathe. This shaft can move freely to or from

* Black, G. V. Appendix Chap. 1, pp. 381–401 in Noyes, F. B. Dental Histology and Embryology, Ed. 2, Phila., 1915.

Patten, B. M. and Chase, S. W. *Anat. Rec.*, 1925, xxx, 123.

Boedecker, C. F. Fundamentals of Dental Histology and Embryology, N. Y.. 1926, p. 228.

† Patten, B. M., and Chase, S. W. *Loc. cit.*, p. 137.

the stone. The other end of the shaft connects with a spiral steel spring. This maintains a constant but slight pressure of the specimen against the grinding surface. The spring can be set to vary the pressure as desired. A set-stop, calibrated to represent the thickness of section in micra, can be adjusted so that when the desired thickness is reached the spring no longer acts and grinding stops. The section is then removed from the carrier by some balsam-solvent, polished by hand, and mounted as desired.

In these machines, the lathe should be heavy in order to minimize vibration and its parts should permit as little play as possible. An abundant supply of cold, preferably iced, water should flow over the stone and the specimen.

An objection to the grinding method as outlined above is that only one or two sections in parallel planes can be prepared from a tooth; however, several longitudinal sections can be obtained from one specimen approximating the human tooth in size, by the use of various slicing machines devised for this purpose. Black* recommends a motor-driven aluminum disc (copper is frequently used) (24 to 30 gauge and about 3½ inches in diameter). The tooth to be sliced is fixed in a brass tube slotted at the free end, with plaster of Paris or sealing wax. This tube is clamped into an object holder fixed upon the slide rest of the lathe. The object holder may be swung horizontally to any possible position in relation to the aluminum disc. When set, the object is moved slowly against the revolving disc, which is fed with carborundum powder suspended in soapy water.

3. Petrifaction Method. The methods of grinding sections so far considered permit only the examination of the hard substances of the tooth. It is obviously important for some purposes to have a method which will preserve the hard and soft dental tissues in their natural relations. In most instances this is accomplished by decalcification which, however, destroys in toto mature enamel. When it is desired to preserve all the dental tissues including enamel in one section, the only method available is that of petrifaction. This is usually known as the Koch-Weil process, of which several modifications have been described.

The object is cut by a saw or a slicing machine into sections as thin as the technician believes it to be possible without distorting the tissues. These slices are then fixed and stained in bulk. Grenacher's alcoholic borax-carmine for a fortnight or aniline blue-black have been recommended.† After staining, dehydration is accomplished by gradually increasing strengths of alcohol. After removal from absolute alcohol, the pieces are cleared in oil of cloves or oil of cedar wood for twelve hours. The oil is quickly washed off with xylol and the pieces are suspended in pure chloroform for twenty-four hours. Then they are put in chloroform balsam for a day, when more dry balsam is added until the chloroform can

* Black, G. V. *Loc. cit.*, p. 400.

† Hopewell-Smith, A. Dental Microscopy, Ed. 3, Phila., 1914, p. 48.

take up no more. This occupies three to four days. Finally the container (an individual container for each specimen, made of heavy lead foil, is very useful—Boedecker) with the pieces is put over a water-bath whose temperature should not rise above 90°c. A drying oven maintained at this temperature would be better. In two to three days (it may take much longer) when the thoroughly cooled balsam cracks like glass on the introduction of a needle point, the slices are removed and ground to the desired thickness by any of the grinding methods outlined above. The impregnation with balsam can be hastened if it be carried out in a partial vacuum chamber.

4. Decalcification. Decalcification with hydrochloric or nitric acids is widely used to prepare specimens consisting of hard and soft tissues so as to retain the natural relations of their parts. The chief, or only, disadvantage is the fact that this entails the loss of enamel. For most purposes however this is unimportant. Before decalcifying, the pieces should be cut with a fine-toothed jeweler's saw into as thin slices as possible without distorting or tearing the parts to be studied. If it be desired to study the pulp in situ, it is best to grind away the enamel and dentin on a stone, using plenty of water, until the pulp is almost exposed in one or two places. The specimen is then fixed before putting it into the decalcifying solution. A relatively large volume of this should be used, and the specimen should be exposed to its action no longer than necessary.

Seidel* has devised a special jar for decalcification, which seems to possess some advantages. It consists of a glass cylinder which has an outlet tube at the bottom and an inlet tube at top. Its cover is ground to form a tight joint. At half its height the cylinder is constricted to support a perforated glass plate on which the specimen is placed. The decalcifying fluid can be withdrawn and replenished with a minimum of disturbance to the specimen. Likewise the washing and dehydrating following decalcification can be performed.

After decalcification the specimen may be imbedded either in paraffin or celloidin. In general the latter imbedding material is much to be preferred.

5. Celloidin Technique of Williams.† A technique has recently been published by Williams who has had a great deal of experience in this line.

a. Fixing and Hardening.

(1) Fix at body temperature.

(2) Using the fluid of Regaud or Guild, inject through the ascending aorta whenever possible, and fix subsequently for two to four weeks. When injection is not possible, use Zenker's for one to three days, followed by Regaud's or Müller's fixative, or use 4 per cent formaldehyde for one to two months.

(3) For better penetration, open the pulp chamber whenever possible, and reduce the size of the tissues with a jeweler's saw.

b. Removing the Fixative.

* Seidel, *per* Brammer und Brauer. *Deutsche Monatschr. f. Zahnh.*, 1926, xliv, 476.

† Williams, A. *Dental Cosmos*, 1927, lxvii, 715.

(1) Wash in running water for twenty-four to forty-eight hours.

(2) Remove the mercuric chloride or oxide with iodine in 80 per cent ethyl alcohol, both in bulk and in section. If the insoluble red mercuric iodide forms, add some potassium iodide solution.

c. Dehydrating.

Use ethyl alcohol in the following dilutions for twenty-four hours each: 10 per cent, 20 per cent, 30 per cent, 40 per cent, 50 per cent, 60 per cent, 70 per cent, 80 per cent, 90 per cent, 95 per cent, absolute 1, absolute 2, absolute 3. If mercuric chloride is present, add the iodine while the tissue is in the 80 per cent alcohol.

d. Imbedding.

(1) Use for twenty-four hours each, a solution of absolute alcohol 3 parts, plus ether 1 part; then absolute alcohol plus ether, equal parts. Use thin celloidin for at least a week, and after the tissue has been in this for twenty-four hours, remove the air by means of negative pressure. Use celloidin of medium thickness for at least a week, and evaporate to greater thickness in a desiccator.

(2) Use chloroform vapor to obtain the proper consistency and store in 80 per cent alcohol for a short length of time only.

e. Decalcifying. Note this is done after imbedding.

(1) Carry the tissue through 50 per cent alcohol for twelve to twenty-four hours, distilled water a few hours, and decalcify either in 5 or 10 per cent nitric acid. Decalcification may or may not be carried on at 37.5°C., according to the type of tissue.

(2) The relative end-point of decalcification may be determined by a chemical indicator.

(3) Neutralize the acid with 5 per cent sodium sulphate for twenty-four to forty-eight hours, and wash away the sodium sulphate in running water for twenty-four to forty-eight hours.

f. Re-imbedding and Mounting.

(1) Dehydrate with ethyl alcohol of the following strengths for twenty-four hours each; 30 per cent, 60 per cent, 80 per cent, 90 per cent, 95 per cent.

(2) To re-imbed, pass through absolute alcohol for twelve hours, or until the celloidin is soft, carefully dissolve away most of the thin celloidin in absolute alcohol plus ether equal parts, and carefully run in fresh, thin celloidin. After twenty-four hours, air may be removed from the tissue by very judicious use of negative pressure. After at least a week in thin celloidin, the tissue is transferred to celloidin of medium thickness and carried through the same steps as in the primary imbedding.

(3) The hardened block of tissue in celloidin is moistened on one side with absolute alcohol plus ether, equal parts, till soft, then mounted on a red fiber block in a drop of thick celloidin. Harden the whole in chloroform vapor and store for a short time only in 80 per cent ethyl alcohol.

g. Cutting.

Cut with the sliding microtome, using 80 per cent alcohol, and store sections in 80 per cent alcohol.

Notes on the above method:

(a) Regaud's fixative.

Potassium bichromate	25 gm.
Sodium sulphate	10 gm.
Distilled water	1000 c.c.

Before using add 20 c.c. of 40 per cent (actual) formaldehyde to every 100 c.c. of fixative.

(b) Guild's fixative.*

* Guild. *J. Lab. & Clin. Med.*, 1919, iv, 153.

(c) The end-point of decalcification. This is usually roughly determined by the flexibility of the tissue or by sticking a sharp needle into it. Williams suggests a better way: the test for phosphorus* giving the ammonium phosphomolybdate precipitate, is applied daily to the successive changes of decalcifying solution until the test is negative.

Pond has suggested that on successive days the decalcifying solution be titrated for loss in acidity and that this loss be made good by the addition of concentrated acid. If a volatile acid like hydrochloric is used it would be necessary to be sure that the loss in acidity is not due to this volatilization. Under this condition, the end-point of decalcification would be indicated by the fact that on titration there was no depression of the acidity of the solution.

Williams has found that for clearing and mounting reagents, the creosote made from beechwood† by E. de Haën of Hanover, Germany, and has been most satisfactory. This does not tend to decolorize stained sections. For clearing after Van Gieson stain, the real oil of origanum-creotic should be employed. Because of the neutral reaction of white euparal and its other desirable qualities, it is now widely used as the best mounting medium.

6. Dextrin Imbedding and Frozen Sections. Instead of imbedding in celloidin the tissues may be imbedded in a dextrin solution and cut on the freezing microtome. This method has been developed by Hopewell-Smith.‡ In experienced hands it yields beautiful isolated sections and in time it is somewhat shorter than celloidin imbedding. However, serial sections and exact control of thickness are impossible. After fixation the soft parts and apices of the roots are dried on a cloth, and a large drop of thin celloidin placed on them, so that in a few moments a thick film covers them over, and protects them from the action of acid reagents, e. g., as in decalcification. Afterward the celloidin film is removed by immersion in a watch-glass of ether for about five minutes, followed by careful picking or rubbing with a brush, and the specimens are neutralized with sodium or lithium bicarbonate (5 grains to an ounce of water). They are then trimmed to appropriate size and shape, washed well in water, and immersed in a saturated solution of dextrin for at least fifteen hours. The pieces of tissue are finally placed on the stage of an ether-freezing microtome and cut in the ordinary manner.

B. EXAMINATION OF SPECIAL STRUCTURES IN DENTAL HISTOLOGY

1. The Enamel-cuticle. By preference take a newly erupted tooth or an impacted one that has just been removed, put in any good fixative, wash and immerse in a weak, decalcifying solution. At intervals of about two

* Treadwell, F. P., and Hall, W. T. Analytical Chemistry, Ed. 4, N. Y., 1915, Vol. II, p. 446.

† Available in the American market.

‡ Hopewell-Smith, A. *Loc. cit.*, p. 55.

hours examine. As the enamel goes into solution, there will be seen surrounding the crown of the tooth, a delicate, whitish, opaque membrane. This can be teased off with needles and a brush. It can, upon removal, be treated by any of the ordinary methods for staining and mounting. After aqueous staining it may be mounted directly in Farrant's medium (p. 473) for examination.

Nasmyth's membrane, when present, is also demonstrable by Boedecker's method (see below).

2. Isolated Enamel Rods. These can be simply demonstrated by immersing the tooth in 5 to 10 per cent hydrochloric acid solution. Examine after twenty-four hours. If the enamel is quite soft, remove a small portion with a needle to a slip, and tease it out. Mount in a drop of physiological salt solution under a cover glass. A carmine stain can be run in from the side and later be washed out by applying a blotter to one edge of the cover glass and running acidulated water (10 per cent acetic) in from the other side. A permanent preparation may be then made by "ringing."

The structure and relations of the individual enamel prism have long been studied by direct microscopic observation of ground sections during the progress of decalcification.*

Dilute acids are rum "under a coverslip supported on fine cover-props over a thin ground section of enamel. The acid can be continuously changed by employing a piece of blotting paper at the opposite side of the coverslip from that at which the acid is supplied. One per cent nitric, hydrochloric, or sulphuric acids or 5 per cent chromic, acetic, citric, or lactic acids decalcify slowly enough so that all the steps in the process of decalcification can be observed without gas being formed sufficiently rapidly to obscure the picture. The process may be stopped at any stage desired by supplying water instead of acid, and, if sufficient care be used, the section can be stained and mounted in balsam without disturbing the position of the remains."

3. Structure and Chemical Composition of Enamel.

Boedecker's Method.

Boedecker† has devised a "celloidin-decalcifying method" to demonstrate minute quantities of organic matter in the enamel.

A small piece of enamel (0.5 to 1 mm. thick), entirely freed from dentin, is passed through the alcohols for dehydration, ten minutes each; then into methyl alcohol for one to two hours and finally into acid celloidin (parlodion) (20 c.c.). This is prepared as follows: Thoroughly dried chips of parlodion (Dupont) are dissolved in c.p. methyl alcohol to make a thick syrupy solution. To 150 c.c. of this add the following mixture drop by drop, constantly stirring with a glass rod:

Nitric acid, C.P.	10 c.c.
Methyl alcohol	40 c.c.

The glass dish in which the enamel is decalcified should have an air-tight cover. This dish should be moved as little as possible and then only

* Chase, S. W., *Anat. Rec.*, 1927, xxxvi, 239.

† Boedecker, C. F. *Loc. cit.*, p. 223.

with the greatest care. The progress of decalcification should be checked occasionally microscopically without removing the enamel from the acid-celloidin. The organic matrix of the enamel will become discernible after ten to twelve hours as a brown spongy substance.

When the entire specimen has acquired this appearance, decalcification is complete, usually within two to seven days. Then uncover the dish to allow the celloidin to harden. Cut out specimen, surrounded by a narrow margin of celloidin and pass this block into 70 per cent alcohol, one to two hours; 40 per cent alcohol, one to two hours; watery solution of alum, twenty-four hours; running water, six to twelve hours; ascending alcohols, one to two hours in each; do not use absolute; aniline oil, six to twelve hours (when cleared block becomes brown and perfectly transparent); aniline, chloroform, equal parts, three to six hours; chloroform, six to twelve hours; imbed in paraffin (m.p. not above 52°C.) two hours, cut sections on microtome, 3 μ to 10 μ; spread sections on slips; dry; decerate in xylol, three minutes; dissolve parlodion in ether-alcohol (ethyl), equal parts; pass through absolute alcohol, one minute; through descending alcohols to water; stain (Boedecker prefers Heidenhain's ferric hematoxylin); dehydrate; mount in balsam.

Chase* has used Boedecker's or Malleson's method to study enamel prisms and interprismatic substance. He, however, shortened the period of decalcification to about one-quarter (or less) of that described.

4. The Structure of Enamel. In relation to its organic content, its permeability and the possibility of post-eruptive changes this is one of the most important questions in dental histology. A number of methods have been devised to attack this problem.

a. Immersion of Apex of Tooth in Stain. V. Beust† cemented freshly extracted teeth in the corks of bottles containing a strong alcoholic solution of fuchsin with NaCl to accelerate capillary flow, in such a manner that only the tip of the root reached the stain. In time the enamel, and in many cases the enamel cuticle, became stained.

b. Injection of Stain into Pulp Canal under Pressure. He also performed the following experiment:

The nozzle of a syringe is securely cemented into the enlarged apical opening of a root with a very large pulp cavity, from which the pulp has been removed, and is completely filled with staining solution. A wire is repeatedly inserted and withdrawn in order to expel all the air. With the fingers, or with rubber bands, pressure is exerted upon the piston. In a short time, varying from a few minutes to a few days according to the pressure and the density of the tooth, the staining solution will be perceptible in all parts of the external tissue. Ground sections are made of such teeth.

c. Silver Nitrate ($AgNO_3$). Later, v. Beust‡ used silver nitrate as the "staining" solution. He proceeded as follows:

The root of a freshly extracted tooth is sawed through about midway between the apex and the crown, and the root and pulp cavity enlarged to receive the end of a glass funnel or pipette. The surface of the root is superficially ground, up to the enamel line,

*Chase, S. W. *Loc. cit.*, p. 272.
† v. Beust, T. *Dental Cosmos*, 1912, liv, 659.
‡ v. Beust, T. *Dental Cosmos*, 1914, lvi, 201.

and the sawed end and ground surface shellacked, care being taken to prevent the varnish from reaching the inner part of the pulp cavity or the surface of the crown. The funnel, containing a wire, is now sealed into the tooth with sticky-wax, allowing the wax to spread over the shellacked part of the tooth. After the wax has cooled, a few drops of water are placed in the funnel and the wire withdrawn (to exclude the air). One per cent aqueous silver nitrate solution is now added and the specimen is placed in the dark. After the lapse of a few days the tooth is ground in paraffin oil, the section is wiped and placed in a bottle containing dilute alcohol. The bottle is now placed in the sun until decomposition of the salt has taken place, whereupon the specimen is passed through strong and finally through absolute alcohol, washed in xylol and imbedded in balsam.

d. Williams' Silver Nitrate ($AgNO_3$). Williams* has studied the enamel very carefully for many years.

An entire tooth is immersed in a 2 to 5 per cent solution of silver nitrate and left in it from one week to six months. Long-continued staining will often reveal facts not shown by brief staining. The tooth is then cut, ground and polished as previously described (p. 265) to the thickness of a single enamel rod.

During the grinding the specimen must be examined from time to time in the following way: Clean it and brush it over for thirty seconds or so with a 3 per cent solution of lactic acid. This will remove a little of the cement substance between the rods and also around the organic substructure in the rod, thus revealing something of the real nature of the tissue. Wash off the lactic acid, cover with a drop of distilled water and cover glass, and examine.

All study and all photography should be done with distilled water for a mounting medium. Permanent preparations may be made by mounting in balsam.

e. Staining Enamel in Bulk. Attempts have been made to stain enamel in bulk.

For example Leigh† has obtained the best results from the use of freshly extracted teeth, eliminating formalin from the technique entirely, passing them through graduated solutions of alcohols, and then immersing them in one of the following stains: (1) 2 per cent alcoholic solution of basic fuchsin or gentian violet or equal parts of crystal violet and brilliant green: (2) 10 per cent aqueous solution of c.p. silver nitrate. The roots of the teeth were cut off to permit free entrance of solutions into the pulp chamber, and the solutions diffused through the dental tissues. Resection of the root also obviates a high liability of the microscopic sections to longitudinal fracture. A varnish of celloidin was used on the exterior of the enamel in certain cases to prevent the tooth from being subjected to stains from both surfaces. After the teeth had been in the staining solutions for varying periods of time, some were bisected longitudinally, and others cut in cross-section blocks, and then ground approximately 10 μ on Black's microlethofer, under either water or paraffin oil. Great difficulty was encountered in

* Williams, J. L., *Dental Digest*, 1925, xxxi, 827.

† Leigh, R. W. *J. Am. Dent. Assoc.*, 1925, xii, 1415.

preventing decolorization of the blocks during mounting, grinding, and demounting.

f. Intra-vital Dyes. Use of "intra-vital dyes" in studying formation and structure of enamel and dentin.

(1) Madder Feeding. Gottlieb* fed a series of dogs, from the beginning of tooth-eruption for three months, on madder. Ground sections made from the teeth of these animals showed that red-colored calcium had been deposited in dentin, the dentino-enamel junction and enamel.

(2) Sodium Alizarinsulphonate. In the same report Gottlieb describes an experiment on 2 dogs, 1 very young and 1 about a year and half old. They both were injected subcutaneously and intravenously with a 1 per cent solution of sodium alizarinsulphonate. Teeth taken from these dogs showed in the dentin adjacent to the pulp an intense violet coloration. Toward the periphery the intensity decreased but nevertheless the color did actually extend into the enamel.

(3) Trypan Blue. Similar work was carried on by Wellings.† Animals were injected with the maximum dose (1c gm. to 20 gm. of body weight) of color solution at intervals of seven or eight days, until the desired degree of staining was reached, when they were killed, and the tissues instantaneously fixed by introducing 10 per cent formalin in normal saline through the heart. The solution of the dye was made in physiological circulating fluid, at first according to Ringer's formula, but later in Schiassi's fluid.‡ It was always boiled in a sterile test-tube before use. Injections were made under the strictest aseptic precautions. In the younger animals the entire head, without decalcification, was impregnated with gelatin according to Gaskell's method.§ In this method, the gelatin is formalinized to render it insoluble, after which fairly thin sections can be obtained by freezing the gelatin block with CO_2 and cutting on the Aschoff freezing microtome.

Older heads were decalcified with 5 per cent hydrochloric acid, with the addition of 1 per cent formalin to preserve the tissues. On the completion of decalcification the tissues were washed in tap water. Prolonged washing is undesirable. Then they were taken through the alcohol series (a little formalin added to all the dehydrating solutions prevents the washing out of the stain) into alcohol and ether, and thence into celloidin. In order to examine for finer cytological details it was necessary to imbed in paraffin. Here again all the dehydrating solutions should carry a little formalin with them. Ground sections were also prepared. In order to keep the section cool while grinding without decolorization dilute hydrochloric acid (1–1000) was found most satisfactory. Small fragments of soft tissues, i. e., pulp, were examined by teasing out in a drop of Farrant's solution.

* Gottlieb, B. *Ztschr. f. Stomat.* 1913, xi, 452.
† Wellings, A. W. *Trans. 6th Internat. Dent. Cong.*, Lond., 1914, p. 46.
‡ Schiassi, B. *La Semaine Méd.*, 1913.
§ Gaskell, *J. Path. & Bacteriol.*, 1912, xvii, 58.

By this method the matrix of dentin and bone is stained very lightly, the color disappearing quite soon. The walls of the dentinal tubules and the Haversian canals do not stain, neither do the dentinal fibrils. The secondary dentin, formed in the center of the pulp cavity of persistently growing teeth, stains deeply. Enamel developing at the time of the introduction of the stain, and that formed while the stain is in the body, takes on a very brilliant color which subsequently fades as decalcification progresses. It is not possible to stain already calcified enamel by means of trypan blue.

(4) Gies. Gies* gave a series of puppies occasional intraperitoneal injections of trypan blue, at different stages of the growth and dentition of the dogs, and found that when trypan blue had been injected, after the permanent teeth had attained their full size, the teeth did not take the stain. When the trypan blue had been injected after the development of the permanent teeth had been far advanced, but before attainment of full size, the teeth showed a narrow blue zone above the gum line, extending about one-fourth the distance to the occlusal surface. When the trypan blue had been injected, soon after the permanent teeth had begun to form, the teeth showed a wide blue zone extending about three-fourths of the distance from the gum line to the occlusal surface. When trypan blue was injected before the permanent teeth had begun to form, the teeth were blue over the entire surface of each crown.

In preparations from other dogs similarly treated the blue pigment was seen in both dentin and enamel.

(5) Marshall.† Marshall extended this work, using the following method:

> The dyes which seemed to give the best results were naphthamine brilliant blue and trypan blue, employed in 1 per cent aqueous solutions and in 1 c.c. doses. The intervals between the injections were purposely arranged at one, two, three, four and five days. All solutions of the dyes, and the injections, were made under the strictest aseptic precautions. Immediately after killing the animal the head was placed in a glass container and covered with warm 10 per cent formalin solution. This was kept in an electric oven at a temperature of 35°C. for from three days to one week. Upon removal from the oven, both jaws were dissected from the head, and each divided on the median lines, the parts again placed in warm aqueous solution of formalin, and returned to the oven at the previous temperature. Later, the parts were passed through the alcohols, from 40 per cent to absolute, increasing 5 per cent each successive twelve hours and then placed for from one to three weeks in benzol-damar, at room temperature. When the specimens were thoroughly impregnated with damar, they were transferred to the discs of a grinding machine, cemented in place with thicker benzol-damar, and again placed in an electric oven, this time at a temperature of 50°C., and allowed to remain for two or three days, or until the damar was sufficiently hardened to prevent the specimen from being torn from the disc in grinding.

* Gies, W. J. *J. Nat. Dent. Ass.*, 1918, v, 529.

† Marshall, J. S. *J. Dent. Res.*, 1921, iii, 241.

By this method, Marshall demonstrated definite color lines in the dentin. Alizarin sodium sulphonate is the only red vital dye studied, that possesses the power to stain the dentin with definite color lines which can be seen under the microscope.

(6) Trypan Blue, India Ink, Methylene Blue. Fish* injected intravenously or subcutaneously trypan blue and iron solutions for the study of dentin.

(7) India Ink. In a later experiment a young dog of a large breed was anesthetized and part of the crown of one of the canines was sawed off to expose the pulp. A syringe filled with India ink and fitted with the finest ordinary hypodermic needle was introduced along the wall of the pulp chamber to about half-way down the root, and while it was being slowly withdrawn the ink was allowed to leak out. No pressure whatever was used. After injection the pulp chamber was not sealed and the dog was killed after an hour or so. Then the tooth was cut in half and the ink was found to have penetrated the dentin. Sections were made to study the details of distribution of the carbon particles.

(8) Methylene Blue. In a continuation of this study Fish† anesthetized an adult dog, drilled a cavity in the crown of one of the teeth near the neck and freely exposed the pulp. A small quantity of solid methylene blue was introduced into the cavity and gently worked into the pulp with a probe, without destroying the tissues of the pulp. A trace of moisture was then added to the dye to ensure its solution in the lymph of the pulp and the cavity was covered with a smear of temporary cement. The dog was kept under morphia and was killed from twelve to twenty-four hours later. On examination a wide zone of a dense blue color was found around the part of the tooth at which the dye was introduced, and where it had entered, the enamel had the appearance of dark blue Bristol glass. Teeth so prepared may be preserved in 10 per cent formalin indefinitely and sections made by grinding. After they are ground the sections may be immersed in 6 per cent ammonium molybdate solution for twenty-four hours, then freshened up on the hone and mounted in Farrant's medium.

5. Examination of Ground Sections of Teeth by Polarized Light. Kirk‡ and others have examined ground sections of teeth through the microscope by means of polarized light. Differences in the degree of calcification are thus easily recognized. The details of the technique have never been given; and unfortunately little has since been done along this line.

6. Examination of Ground and Decalcified Sections of Teeth by Ultraviolet Light. Walkhoff§ has applied ultraviolet light as a source of illumina-

* Fish, E. W. *Proc. Roy. Soc. Med., Sect. Odont.*, 1926, xix, 59.
† Fish, E. W. *Proc. Roy. Soc. Med., Sect. Odont.*, 1927, xx, 1.
‡ Kirk, E. C. *Dental Cosmos*, 1903, xlv, 345.
Gerhardt. *Arch. f. Entwicklungsmechanik*, x, 135, 263.
§ Walkhoff, O. *Dental Cosmos*, 1923, lxv, 65.

tion for the microscopic examination of sections of teeth. Of course the lenses, slip and coverslip must be of quartz and the record is made on a photographic plate. Direct examination by the eye is impossible. Because of the shorter wave-length, this method possesses distinctly greater resolving power. Walkhoff was primarily interested in the problem of an interprismatic, cement-substance in the human enamel. The details of the technique are rather elaborate and are given in the original article.

7. Examination of Ground Sections of Teeth by Roentgen Rays. Two interesting papers have just appeared* which may open the way to a more refined analysis of the structure of enamel and dentin than has hitherto been possible. Laue's method of making roentgenphotograms was employed. The source of the roentgen rays was a Coolidge electron tube with a molybdenum anticathode. The radiation lasted from fifteen to fifty hours. The sections of the teeth were 1 to 0.3 mm. thick. In studying the dentin the directions of the rays were (a) at right angles to the dentinal fibers and (b) parallel with the dentinal fibers.

Cross, longitudinal and tangential sections of human incisors and molars of cattle were used in the study of the enamel. The sections were 1 to 0.4 mm. thick. They were so placed that a narrow bundle of roentgen rays would pass (a) parallel, (b) at right angles vertically, and (c) at right angles horizontally to the long axis of the enamel prism. The dentin had previously been ground away and the upper surface of the enamel section had been etched with dilute HCl in order to exclude the influence of the grinding on the arrangement of the superficial microcrystals.

8. Examination of Ground Sections of Teeth by Reflected Light (Opaque Illumination). A type of microscope often employed in mineralogical studies is used.† It is very important to have a brilliant source of illumination, as an arc light, exactly centered to cast its beam horizontally through a lateral opening in the tube of the microscope onto a prism. This prism lies within the tube and is adjusted at such an angle that the light is thrown down onto the object. The light is reflected therefrom back up the tube to the eye of the examiner. An advantage of this method of examination is that it renders unnecessary the tedious preparation of thin ground sections. It is however absolutely necessary that the surface of the tooth be very highly polished and be placed parallel to the stage of the microscope. Beautiful photomicrographs, even at a magnification of 1600 diameters, have been made in this way.

A somewhat similar method has been devised by Köhler and Sonnenburg,‡ which, however, does not require the special mineralogical or metallurgical microscope.

* Funaoka, S. *Acta Scholae med. univ. imp., Kioto,* 1926, ix, 37 and 41.

† Friedeberg, *Deutsche Monatschr. f. Zahnh.,* 1922, xl, 57.

‡ Köhler and Sonnenburg, *Vrtljbrschr. f. Zahnhlk.,* 1923, xxix, 230.

(1) The tooth to be examined may be fresh or fixed. It may have been subjected to vital or supravital staining, or it may have been prepared by the Koch-Weil petrifaction method. Sometimes it is desirable, in order to prevent too deep a penetration of the stain after grinding, to cover it with a thin layer of some transparent material, as an acetone-celloidin solution.

(2) The tooth is then set in a plaster-of-Paris base, whose under surface determines—is parallel to—the plane of grinding. Enough of the tooth should project above this base to permit the focusing of a beam of light within the body of the tooth. The base is conveniently prepared as follows: Cut a section of rubber tubing, 1 inch or more in diameter and as long as the tooth in question. Mix a thin batter of plaster and pour it into the tubing which rests on a glass slab. The plaster should come up about half way in the tubing. The tooth should be inserted into the plaster in such a manner that the plane of the glass slab is parallel to the plane through the tooth, which it is desired to examine.

(3) The tooth is held against a revolving grinding stone and ground down to the plane which it is desired to examine. It is most essential that the base be held in a plane parallel to the stone. This can be easily done by the use of various mechanical devices.

(4) The ground surface is carefully and thoroughly polished on an Arkansas stone with aluminium oxide.

(5) This surface is etched five, ten or twenty minutes with 6 per cent nitric acid, which is washed off with water.

(6) The acid is neutralized by ammonia vapor. The block with the tooth is placed on a piece of blotting paper moistened with ammonia water and covered with a small bell-jar. One can now immediately pass to (10) below or he can apply a stain.

(7) Apply Delafield's hematoxylin to ground surface, two to five minutes.

(8) Differentiate in distilled water to which a little acid is added (*ca.* 1: 20).

(9) Repeat (6). A shorter time will suffice.

(10) Wash in increasing concentrations of alcohol, then carbol-xylol, and attach a cover glass to the ground surface with Canada balsam. It is sometimes desirable, especially if high magnification be desired, to clear the entire tooth in glycerin or by the Spalteholz method before attaching the cover glass.

After the balsam has hardened, the plaster base is broken off and the tooth is suspended by the cover glass into a small glass cylinder.

The specimen is examined by the following manner of illumination: Horizontal rays from a carbon arc are filtered through water, and collected by a biconvex lens which focuses them to a point within the substance of the tooth. This point lies in the optical axis of the microscope and below the ground surface to be examined. This ground surface forms an optical plane or section which is examined in the usual way by the microscope.

Any number of such optical planes in a given tooth may be studied and photographically recorded, by simply removing the cover glass and grinding down to a new surface and repeating (4) to (10) of this method.

9. Interglobular Spaces. These can usually be seen in unstained ground sections of teeth, particularly when they have been dried before mounting in balsam. However they can be made more conspicuous in at least three ways.

(1) When the ground section is 0.5 mm. or slightly less in thickness, let it stand overnight in the dark in a 1 per cent aqueous solution of silver nitrate. Remove, rinse in distilled water, and then immerse in 1 per cent formalin in direct sunlight until the specimen becomes deep black. Then finish grinding to desired thickness, polish and eventually mount in balsam.

(2) Stain ground sections in a hematoxylin solution for a few minutes, partially decolorize with very dilute acetic acid. Control this under the microscope. Rinse in distilled water as soon as only the interglobular spaces retain their color: then into 1 per cent osmium tetroxide (osmic acid) for one hour. Wash, clear and mount.

(3) (*Charters White's Method.*) Sections of teeth, *ca.* 1 mm. thick, are dehydrated, passed through ether and absolute alcohol (equal parts), and then into a stained celloidin solution for several days. The celloidin solution is made as follows: add fuchsin to absolute alcohol until a "dark port wine color is produced," mix this with an equal part of ether and add celloidin to required consistency. Remove sections and dry by evaporation. They may be kept indefinitely in this condition. When desired they are ground to the required thickness, polished and mounted in balsam.

10. Lymphatics of Pulp. *a. Schweitzer** injected the blood vessels with a carmine mass and the lymph vessels with Berlin blue-turpentine-ether mixture, according to the method of Gerota.†

The animals were either killed by chloroform and immediately injected; or after rigor had passed were warmed to about 50°C. The injection was made into the common carotid or in the case of the smaller animals into the ascending aorta after opening a vein. The pressure must be maintained for some time in order to insure the injection of the gingival capillaries as indicated by the reddening of the mucosa. The lymph vessels were injected by the same method as that used for macroscopic studies, only with great care not to use too strong pressures. Schweitzer assumed that the lymph vessels of the pulp, if such exist, probably communicate by anastomosis with those of the perodontal tissue. Therefore, by injecting the lymph vessels of the gingiva near the teeth, it could be expected that under favorable conditions one might force the fluid from these into the lymphatics of the pulp, regardless of a flow in a retrograde direction. In this way Schweitzer actually succeeded in filling lymph vessels in the pulp in some cases. Schweitzer's experience leads him to a conclusion that when the purpose of the studies is to reveal the distribution of the lymph vessels, it is desirable to have the injection of the lymph vessels precede that of the blood vascular system. The tissues were fixed and hardened in formalin (alcohol or Müller's fluid, diluted 1:10), decalcified in 1 per cent hydrochloric acid, imbedded in parlodion and serially cut at 200 μ.

b. Dewey and Noyes. The work of Dewey and Noyes‡ was done chiefly on dogs. In all instances the blood vessels were injected with carmine gelatin in order to eliminate confusion as to whether the channels were lymph vessels, or arteries and veins. After ligation of the superior vena cava and the arch of the aorta in two places and opening the jugular vein or the vena cava, the cannula was inserted into the left common carotid. The vessels were thoroughly washed out with physiological salt solution and the carmine gelatin mass injected. The body was cooled off under running

* Schweitzer, G. *Arch. f. mikr. Anat.*, 1909, lxxiv, 927.

† Gerota, *Anat. Anz.*, 1896, xii, 216.

‡ Dewey, K., and Noyes, F. *Dental Cosmos*, 1917, lix, 436.

water for from five to ten minutes. Before beginning with the injection of the lymph vessels, some time was allowed to pass, to insure the complete solidification of the gelatin. The head was severed, the fur removed, and the head warmed in warm water. They used the injecting mass which is now most frequently employed, i. e. the Gerota mass. For this, 2 gm. Prussian blue (oil color in tubes) is stirred with 3 gm. turpentine oil in a glass mortar for five minutes, 15 gm. of sulphuric ether is added and this fluid is filtered through flannel or chamois skin. They used ordinary fine steel needles with Luer's syringe. This facilitated keeping the injecting point as near the deep portions of the teeth as desired. While injecting, slight pressure was made with the finger over the place of the inserted needle, and after withdrawing the latter, gentle even massage was continued for some time. All excess of the fluid on the external surface was carefully washed off. The head was left on the table for an hour or longer, and thereupon placed in 20 per cent formalin. After twenty-four hours, or at any later period, the injected teeth were singly sawed out from the jaw, carefully opened, and the pulp removed and examined.

Another method for injecting the lymphatics of the dental pulp was used by Dewey and Noyes and called by them "the direct method." Because the individual injection of a number of teeth consumes considerable time, that of the blood vessels was made first. A hole was made in the tooth, reaching down to the pulp, and a metal cannula made for this purpose was inserted. Injections were also made with Luer's syringe and a steel needle, the point of which had been filed off to the base of the needle. Both the metal cannula and the syringe barrel were filled with a few cubic centimeters of Prussian blue by means of a medicine dropper. Pressure was exerted in the syringe with the piston over a column of air, in the metal cannula, through a pressure tank or by blowing with the mouth for longer or shorter periods into paper cones, the tapered ends of which were inserted into the cannula. Unless the needle or the cannula fits absolutely into the hole the fluid invariably flows back, at even very slight pressure. Special cannulas were made with a conical point to fit tightly in a hole made by a drill with a special thread; with these there was no backward flow. In using the pressure tank, jerky movements produced by compressing the rubber tube in quick succession seemed to be more effective than an even pressure.

Finally, the order of procedure was reversed, and the injection of Prussian blue was made before the blood vessels were filled and distended with carmine gelatin, as it was evidently this condition which rendered the tissues so unduly resistant. The heads and necks of freshly killed dogs were kept warm by constantly applying hot wet cloths to them. Pressure was again effected by blowing or by the air-tank so applied as to bring about the above-mentioned jerky movements. The injection of the blood vessels followed some time later. The results obtained with these procedures, although applied only on a few dogs, were positive in every case, and far

superior to any of the previous ones. The blue injection mass had filled lymph vessels emerging from the maxilla and the mandible and passed in considerable quantities into the submaxillary lymph glands and deep cervical glands.

Supplementary studies of the lymphatics of the pulp were made by an entirely different manner of investigation. A large number of rabbits and also a few dogs and cats were injected intravenously or intraperitoneally with trypan blue or lithium carmine for various periods of time. In these, the blood vessels of the head were well injected with carmine or Berlin-blue gelatin, after which the tissue was put into 10 per cent formalin. After hardening, the pulps of the incisors were removed and frozen sections were made. This method gave suggestive but not conclusive evidence that lymphatics are present in the dental pulp.

11. **Innervation of Pulp and Dentin.** *a. Huber** used "intra vitam" staining with methylene blue to differentiate myelinic and amyelinic nerves in the pulp of the rabbit. Kill the animal with chloroform, insert cannula into common carotid, inject enough 1 per cent methylene blue in physiologic sodium chloride solution to pigment deeply tongue and lips. In half an hour remove mandible and wipe with a clean dry cloth. Remove molar tooth with aid of bone-forceps. Cut away dentin on anterior and posterior surfaces. Insert needle under one of the processes of the pulp and with slight traction carefully remove pulp with as little laceration as possible. Place pulp on slip moistened with physiologic salt solution. In a few moments the axis cylinders are found to be stained deep blue. For permanent preparations fix with a saturated aqueous solution of ammonium picrate or ammonium molybdate: then mount in glycerin. Sections can be cut by placing the pulp between 2 flat pieces of elderpith.

b. Dependorf† used, successfully in his opinion, the methods of Loewit, Bielschowsky and Held to demonstrate the entrance of nerves from the pulp into the dentin and their course in this tissue.

He also studied the distribution of the nerves in the human alveolo-dental periosteum.‡ His methods, given in detail, were applied to teeth immediately after removal from the body and cut into small pieces. Different stain methods were employed: (1) the Cajal method, (2) the Bielschowsky method, (3) the Golgi method, (4) the Loewit method. A modification of the Loewit method gave the most beautiful results. The details of these methods as used by Dependorf follow:

(1) Cajal Method. (a) Place small pieces in 1.5 to 3 per cent silver nitrate solution at 37°C. and keep in the incubator at 37°C. for from three to five days.

(b) Rinse quickly in distilled water at 37°C.

(c) Reduce in pyrogallol formalin solution.

* Huber, C. *Dental Cosmos*, 1898, p. 84.

† Dependorf, T. *Deutsche Monatschr. f. Zahnh.*, 1913, xxxi, 377.

‡ *Ibid.*, xxxi, 853.

(d) Frozen sections may be made, or, in case of calcified sections, decalcify in 5 to 10 per cent formic acid.

(e) Imbed in celloidin and eventually, after sectioning, stain with the Knal silver solution (ammoniacal silver solution) according to Bielschowsky (thirty to fifty minutes).

Small pieces can also after preliminary staining with silver nitrate be quickly washed and then counterstained with the Knal silver solution for one to two hours at 37°C.

(2) Bielschowsky Method. (a) Fix small pieces in 10 per cent formalin up to fourteen days or in 60 per cent alcohol plus 40 per cent formalin, 90: 10.

(b) Wash for from one to one and one-half hours in distilled water.

(c) Cut frozen sections. The smaller pieces may also be stained further in toto and later cut.

(d) Wash in distilled water, two to eight hours.

(e) Immerse in 2 per cent silver nitrate for twenty-four to forty-eight hours or in the case of larger pieces up to four days.

(f) Wash in distilled water, twenty-four hours.

(g) Stain in the Knal silver solution according to Bielschowsky, thirty to sixty minutes; in the case of larger pieces, two to three hours at 37°C.

(h) Immerse momentarily in 10 c.c. of distilled water plus 2 drops of glacial acetic acid. Rinse in distilled water.

(i) Reduce in 20 per cent formalin solution with tap water, for twenty-four hours, and eventually counterstain by gold.

(3) Golgi Method. (a) Fix small pieces in 8 parts of a 2 per cent potassium bichromate solution plus 1 part of a 1 per cent osmic acid solution.

(b) After five days rinse in distilled water or in 1 per cent silver solution.

(c) Immerse in a large volume of 1 per cent silver solution for six to fourteen days and keep in semi-darkness.

(d) Absolute or 96 per cent alcohol, one-half to one hour.

(e) Counterstain in 1 per cent gold chloride solution (8 to 10 drops in 10 c.c. of absolute alcohol) one-half to two hours.

(f) Rinse briefly in 50 per cent alcohol and in distilled water.

(g) Carry over into 10 per cent sodium sulphite solution (five to fifteen minutes).

(h) Wash for a prolonged period and counterstain.

(4) Loewit Method. (a) Fix in formic acid solution (1 part formic acid, 2 parts distilled water), five to ten minutes.

(b) Immerse in 0.25 to 1 per cent gold chloride solution from a few hours to one-half a day in the dark.

(c) Carry over into formic acid solution of strength above mentioned in (a), and leave for twenty-four hours in the dark.

(d) Reduce in concentrated formic acid solution (twenty-four hours in the dark).

An alternative procedure is (a) fix at 37°C.; (b) wash quickly in distilled water at 37°C.; (c) immerse in gold chloride solution (10 parts of distilled water, 2 parts of 1 per cent gold chloride) for two to six hours at 37°C., (d) wash briefly at 37°C., (e) immerse in the formic acid solution, of the strength mentioned above in (a), for two days at 37°C.; change once, keep in the dark. (f) Reduce and decalcify in concentrated formic acid for two to three days in the cold and in the dark.

*c. Fritsch** also studied the innervation of the dentin. The method is as follows:

* Fritsch, C. *Arch. f. mikr. Anat.*, 1914, lxxxiv, 307.

(1) Fix in formalin four weeks at least.
(2) Decalcify, Schaeffer's method.
(3) Cut frozen sections, Reich's method.
(4) Stain in methylene blue, Muench's method, or better,
(5) After Bielschowsky's method keep sections twenty-four hours in water.
(6) Treat with pyridine three to four days.
(7) Wash in distilled water, twenty-four hours.
(8) Treat with 3 to 5 per cent silver nitrate, five to eight days in the dark.
(9) Transfer to a silver oxide bath for four to five minutes.
(10) Treat with 20 per cent formalin for reduction.
(11) Wash with acidified distilled water.
(12) Wash thoroughly with distilled water.
(13) Repeat the steps 9 to 12, best results coming from 10 to 12 repetitions.
(14) Tone sections with gold.
(15) Cover with gelatin on a slip by transferring from water to a solution having 10 gm. gelatin to 100 c.c. water.
(16) Dry gelatin and examine sections under oil immersion lens.

12. Form and Ramifications of Pulp Chamber and Canals. *a. The Work of Preiswerk** aroused interest in this subject.

Open the teeth from the occlusal surface into the pulp chamber: put in water for two or three weeks at about 37°C., wash through with hot soda solution, slowly dry in hot air and surround the roots with blotting paper (to prevent plaster entering apical foramen). Imbed in plaster of Paris and insert into each tooth, with white glue, a high cardboard funnel. Carefully warm for a day over a sand or water bath, then slowly raise temperature until Wood's metal in an adjacent dish begins to melt. Then pour molten Wood's metal abundantly into the funnel to secure enough pressure. Gently tap the preparation against the table to help the downward flow of the metal. After cooling, remove plaster of Paris and saw away the excess metal projecting from the tooth. If the metal comes through the tooth and lies between the apical end and the blotting paper, good results may be expected.

Put the specimen for two to three weeks in *ca.* 20 per cent KOH in an incubator, after which the metal core can be easily shelled out. Dry, varnish with very thin Canada balsam and mount.

b. Fischer† substituted celluloid for the metal casting of Preiswerk. The teeth were kept for several weeks in water at 37°C., to macerate the pulps. They were then gradually transferred to acetone solutions and finally injected with celluloid-acetone solutions of increasing consistency. After complete hardening (evaporation of the celluloid solution) the injected teeth were macerated in pure HCl until every trace of organic or inorganic tissue had disappeared.

c. Adloff. Another method has been employed by Adloff.‡ The teeth were first decalcified, then bleached in hydrogen peroxide by adding it to the decalcifying solution, dehydrated, and cleared in cedar-oil. Permanent preparations can be made by immersing the specimens in vials filled with Canada balsam (not xylol- or chloroform-balsam).

* Preiswerk, G. *Oesterr.-ungar. Vrtljschr. f. Zahnh.*, 1901, xvii, 145.
† Fischer, G. *Deutsche. Monatschr. f. Zahnh.*, 1907, xxv, 544.
‡ Adloff, P. *Ibid.*, 1913, xxxi, 445.

This method is applicable to teeth whose canals have been filled with Wood's metal to demonstrate ramifications of pulp. The teeth, after they have been thoroughly cleared of pulp, decalcified and bleached, are invested in a casting-compound and the molten metal forced in by centrifugal force (tooth and investment at room temperature). They should then be clarified.

*d. Fasoli and Arlotta** followed a slightly different procedure. They forced Wood's metal into empty canals of teeth. Some of these specimens were then radiographed while others were placed in a clearing solution (Spalteholz).

e. Moral† opened wide the pulp chamber of the tooth, which was then macerated and briefly bleached in hydrogen peroxide. It was then washed and dried. China ink was introduced after examination with a lens showed freedom from cracks. A fine smooth broach was used to introduce the ink as deeply as possible. Then the teeth were fixed upright on a glass plate and set in a moist chamber. The apices were directed downward so that the ink of its own weight would tend to penetrate more deeply. When the ink came through to the apex (this usually took twenty-four hours) the tooth was removed from the moist chamber, dried, and after two to three days placed in 15 per cent nitric acid for decalcification. Hydrogen peroxide was added to decalcifying fluid for bleaching and the specimen clarified following Krause's method.‡

f. Hess and Zürcher§ have studied this field very thoroughly. Extracted teeth are drilled into and the pulp cavity is exposed, without however penetrating into the root-canals. Then the teeth are placed in a vessel filled with drinking water, and kept from three to four months in the thermostat at a temperature of 37°C., until the canals can be syringed through from the pulp chamber with a solution of soda at a temperature of 40°C. This result might be obtained in half the time if hydrogen peroxide, free from acid, were used. The teeth are washed for twenty-four hours in absolute alcohol, and are then dried for twenty-four hours at the temperature of a living-room.

Then the roots of the teeth are wrapped in blotting paper to the enamel margin, and they are imbedded in plaster of Paris in an ordinary well-fitting vulcanizing flask, so that the plaster of Paris covers the tooth up to the pulp cavity. When the plaster of Paris has hardened, the pulp cavity of each tooth is packed with rubber, the surface rubbed with talc, and the counter-mould made.

After the hardening of the plaster, the flask is opened and the root-canals and the pulp chambers of the teeth are packed as firmly as possible with ordinary red vulcanite. To avoid adherence to the counter-mould

* Fasoli, G. and Arlotta, A. *La Stomatologia*, 1913, xi, 409.

† Moral, H. *Deutsche. Monatschr. f. Zahnh.*, 1914, xxxii, 617.

‡ Krause, R. *Anat. Anz.* xxxiv, 34, 133.

§ Hess, W., and Zürcher, E. Anatomy of the root canals of the teeth. Lond., 1925.

a piece of linen is placed between the two halves. The flask is now put into warm water and boiled for about twenty minutes, and afterwards closed by gradually increasing pressure. When the rubber has penetrated into the root-canals from the pulp chamber, another piece of rubber is put over each pulp cavity, and the flask is then boiled for twenty minutes and again closed by gradual pressure. The flask is then kept for several hours under the press, gradually cooled, and then during one hour vulcanized under a pressure of 7 atmospheres.

When removed from the vulcanizer, and after cooling the flask, the teeth can be removed from the plaster of Paris with the usual precautions, as now the plaster of Paris is worked easily; or, if not yet soft enough, the flask should stand in water for some days, to soften the plaster of Paris.

After the teeth have been rinsed in water, they are dissolved in a solution of 50 per cent pure hydrochloric acid, which can be done in the thermostat at a temperature of 25°C. to 31°C. in a few hours. The corrosion preparation is now rinsed carefully in running water, freed from the excess of vulcanite, and planted in a plaster of Paris block to form permanent preparations.

*g. Barrett** has confirmed and extended these observations by simply following the dental pulp through a series of celloidin sections, stained with hematoxylin and eosin, of human teeth, cut approximately parallel with the occlusal surface.

* Barrett, M. T. *Dental Cosmos*, 1925, lxvii, 581.

METHODS FOR THE INTERCELLULAR SUBSTANCES OF THE CONNECTIVE TISSUES

F. B. Mallory and Frederic Parker, Jr.

Fibroglia fibrils 287. Collagen 289. Elastic fibrils 296. Mucin 298. Amyloid 299.

It is probable that all the intercellular substances of the connective tissues are formed by one protean cell, the fibroblast. It produces fibroglia, collagen and elastic fibrils, mucus, the ground substances of cartilage and bone and, under certain pathologic conditions, amyloid. Reticulum is a term applied to that form or arrangement of collagen which is colored intensely by silver stains apparently simply as the result of a physical condition, namely, separation into single fibrils or minute strands of them by cells, elastic fibrils or fluid.

I. Fibroglia Fibrils

Fibroglia fibrils are in intimate relation with the cytoplasm of the fibroblast, running over its surface and extending out along its processes in the same way that neuroglia fibrils do. They are very delicate, straight or slightly curved, run in parallel lines and are definitely separated from one another. They seem to pass from cell to cell, thus aiding to form a syncytium.

Fibroglia fibrils are most evident when fibroblasts are leading an active existence as in granulation tissue, in the organization of fibrin (thrombi, etc.) in the stroma of cancers and in fibrosarcomas if the latter are not growing too rapidly. In the spleen and kidneys they can readily be demonstrated, but in certain tissues such as the heart, the endometrium, the ovary, etc. they are not in evidence under normal conditions.

In order to demonstrate fibroglia fibrils the tissue must be absolutely fresh for best results, that is, removed surgically and placed in a fixative within one to five minutes after it is taken from the living body. The reason for this is that the fibrils quickly lose their staining properties post mortem.

1. Fixation. The only fixative recommended is Zenker's fluid and the tissue must be cut into thin sections not over 2 to 4 mm. thick. Fair results can be obtained by zenkerizing formaldehyde fixed tissue but the procedure is not to be encouraged.

(1) Zenker's fluid, twenty-four hours.
(2) Running water, twenty-four hours.
(3) Alcohol, 80 per cent, twenty-four hours.

2. Staining. There are two staining methods which are particularly useful and more or less specific for the demonstration of fibroglia fibrils: a, phosphotungstic acid hematoxylin, which led to their discovery, and b,

acid fuchsin followed by permanganate of potassium. Two other stains often bring them out with great distinctness, namely, the eosin (phloxin)-methylene blue method which colors them a bright pink, and the aniline blue collagen stain by which method they are dyed an intense red by the acid fuchsin.

a. Mallory's Phosphotungstic Acid Hematoxylin Stain.

Water	100.0 c.c.
Hematoxylin	0.1 gm.
Phosphotungstic acid crystals	2.0 gm.

Dissolve the hematoxylin by the aid of heat in part of the water and add it after it is cool to the acid dissolved in the rest of the water. No preservative is necessary. The solution requires several weeks in order to ripen, but may be ripened at once by the addition of 10 c.c. of a 0.25 per cent aqueous solution of permanganate of potassium.

Hematein ammonium may be used instead of hematoxylin but requires 5 c.c. of the permanganate solution to ripen it fully at once.

Staining Method. Zenker fixation, paraffin sections. Treat sections with iodine in the usual way in order to remove the mercury precipitate and then extract the iodine by means of alcohol or a 0.5 per cent solution of hyposulphite of sodium and finally get them into water.

(1) Place sections in a 0.25 per cent aqueous solution of permanganate of potassium for five to ten minutes.

(2) Wash in water.

(3) Oxalic acid, 5 per cent aqueous solution, ten to twenty minutes.

(4) Wash thoroughly in several changes of water.

(5) Stain in phosphotungstic acid hematoxylin for twelve to twenty-four hours.

(6) Transfer sections directly to 95 per cent alcohol, followed by absolute. Dehydrate quickly because alcohol readily extracts the red part of the stain.

(7) Clear in xylol and mount in xylol balsam.

The solution stains nuclei, centrosomes, achromatic spindles and fibroglia, myoglia and neuroglia fibrils, fibrin, and the contractile elements of striated muscle blue, collagen (including reticulum), and the ground substances of cartilage and bone varying shades of yellowish to brownish red. Coarse elastic fibrils are sometimes colored a purplish tint.

If celloidin sections are used, clear with xylol from 95 per cent alcohol by means of the filter paper blotting method. Origanum and other oils cause the blue stain to fade.

Fair results may sometimes be obtained after formaldehyde fixation if the tissues are first carried through Zenker's fluid but the method is not recommended.

b. Mallory's Acid Fuchsin Stain. Zenker fixation, paraffin sections.

(1) Stain in a 1 per cent aqueous solution of acid fuchsin overnight in the cold or for one hour in the paraffin oven (54°C.).

(2) Drain slides and differentiate in a 0.1 per cent aqueous solution of permanganate of potassium for forty to sixty seconds. This step must not be prolonged beyond the exact time needed or the section will be decolorized.

(3) Dehydrate in 95 per cent alcohol followed by absolute.
(4) Clear in xylol and mount in xylol balsam.

Nuclei and fibroglia fibrils red, collagen pale reddish yellow, elastic fibrils bright lemon yellow, red blood corpuscles purplish red.

II. Collagen

Collagen consists of exceedingly delicate fibrils which usually present a wavy appearance. They may be more or less widely separated from each other, or united into thin or coarse strands, or fused into fibrillar or homogeneous masses. Reticulum is the term applied to collagen which has been stained by the silver method and this occurs only under certain physical conditions, namely, when the individual fibrils or small strands of them are separated from each other by cells, elastic fibrils or fluid, but not when they are compacted. The appearance of the deeply stained fibrils is so striking in contrast with the lightly staining denser masses of collagen that they have very generally been assumed to be of a different chemical nature without taking into consideration the results obtainable by the application of other staining methods for collagen. Their origin has been attributed by many to endothelial cells or to reticular cells, whatever the latter may be.

1. **Fixation.** Collagen fibrils retain their characteristic staining properties a long time post mortem but absolutely fresh tissue should be preferred for the study of them whenever obtainable. Any fixative may be employed, but Zenker's fluid is recommended over all others. A variety of more or less specific stains is available for demonstrating them. The oldest and most generally useful is Van Gieson's because it works well after all the ordinary fixatives, but it does not stain the fibrils so intensely as is desirable.

2. **Staining.** *a. Van Gieson's Picric-acid Fuchsin Solution.* This was originally made by adding to a saturated aqueous solution of picric acid enough of a saturated aqueous solution of acid fuchsin to give to the fluid a deep garnet-red color. Nowadays it is usual to employ a 1 per cent aqueous solution of acid fuchsin and to add 5 to 15 c.c. of it to 100 c.c. of a saturated aqueous solution of picric acid according to the intensity of stain desired, or the nature of the fixative employed.

Staining method.
(1) Stain paraffin sections rather deeply with alum hematoxylin.
(2) Wash in water.
(3) Stain in Van Gieson's solution for five minutes or more.
(4) Transfer directly to 95 per cent alcohol followed by absolute.
(5) Clear in xylol and mount in xylol balsam.
For celloidin sections clear in origanum oil after 95 per cent alcohol.

The two drawbacks to this method are that the red stain of the collagen is not intense enough and that it tends to fade. This latter fault may be

obviated in the future by employing the new acid fuchsin, Lot #39, recently introduced by French and guaranteed not to fade.

b. Mallory's Aniline Blue Collagen Stain. Zenker fixation, paraffin sections.

(1) Stain sections in a one per cent aqueous solution of acid fuchsin for five minutes or longer.

(2) Transfer directly to the following solution and stain ten to twenty minutes or longer.

Aniline blue soluble in water	0.5 gm.
Orange G	2.0 gm.
One per cent aqueous solution of phosphomolybdic acid	100 c.c.

(3) Wash and dehydrate in several changes of 95 per cent alcohol followed by absolute.

(4) Clear in xylol and mount in xylol balsam.

For celloidin sections use 95 per cent alcohol and clear by the blotting paper xylol method.

Collagen fibrils, the ground substances of cartilage and bone, mucin, amyloid and certain other hyaline substances are stained varying shades of blue; nuclei, fibroglia, myoglia and neuroglia fibrils, axis cylinders and fibrin red; red blood corpuscles and myelin yellow; elastic fibrils pale pink or yellow. If it is desired to bring out the collagen fibrils as sharply as possible, omit the staining with acid fuchsin.

c. Verocay's Stain for Collagen.* Any fixative can be used; and frozen, celloidin or paraffin sections. All sections must be closely attached to the slip before staining in order to prevent shrinking by the mordant, paraffin sections by means of egg albumen in the usual way, and then in addition, like the frozen and celloidin sections, by means of a thin solution of celloidin poured over them after they have been pressed onto the surface of clean, fat-free slips. Harden the celloidin in 80 per cent alcohol.

(1) Wash sections thoroughly in water.

(2) Mordant them in a 1 per cent aqueous solution of chromic acid at 46°C. or thereabouts for ten to twenty-four hours or more.

(3) Wash thoroughly in several changes of water.

(4) Stain in Delafield's hematoxylin (which must not be too old) for one-half to one hour or longer.

(5) Dehydrate in 95 per cent alcohol. Clear by the blotting paper xylol method and mount in xylol balsam.

Collagen is stained dark blue by this method.

d. Mallory's Phosphomolybdic Acid Hematoxylin Stain for Collagen. Zenker fixation, paraffin sections.

(1) Stain sections in the following solution for twelve to twenty-four hours or more, or in the paraffin oven at 54°C. or thereabouts for two to three hours.

* Verocay. *Centralbl. f. allg. Path.*, 1906, p. 942.

Hematoxylin	1.0 gm.
Phosphomolybdic acid crystals	2.0 gm.
Water	100 c.c.

The solution requires several weeks in order to ripen but may be ripened at once by the addition of 5 c.c. of a 1 per cent solution of permanganate of potassium.

(2) Wash in water.

(3) Decolorize and dehydrate in 95 per cent alcohol followed by absolute.

(4) Clear in xylol and mount in xylol balsam.

It is much simpler than Verocay's method and is believed to yield equally good results. Single collagen fibrils are brought out sharply, a deep blue color, but not so well as by the silver method. If a counterstain is desired, place sections first in a 0.1 per cent aqueous solution of acid fuchsin for five to ten minutes and then drain and transfer directly to the hematoxylin solution.

e. Silver Impregnation Methods for Collagen (Reticulum). Bielschowsky* originally devised his silver impregnation methods for the study of neurofibrils, but Maresch† applied these methods to connective tissue for the study of the finer fibrils of collagen, the so-called reticulum. Reticulum fibers are brought out by these methods more clearly and beautifully than by any other and study of such preparations is a revelation as to the complexity and number of these fibers. Bielschowsky's method depends on the reduction by formalin of the easily reducible soluble silver salt formed by dissolving with ammonia the precipitate caused by adding a solution of sodium hydroxide to a solution of silver nitrate. Numerous modifications of his methods have been devised but the basic principles remain the same.

Certain precautions must be taken in carrying out these methods, for, as is well known, silver is easily affected by organic matter. Some of the main points to be observed are given below:

(1) All glassware must be chemically clean. This is best accomplished by treating it with sulphuric-acid-potassium-bichromate cleaning solution, followed by thorough washing with tap water, followed by distilled water.

(2) The distilled water must be free from organic matter. This can be tested by adding a few drops of a silver nitrate solution and watching for a color change or precipitate. Da Fano recommends using water redistilled on potassium permanganate.

(3) All chemicals must be of the highest chemical purity.

(4) In handling frozen or celloidin sections, glass instruments, never metal, should be used.

(5) Certain filter papers contain sufficient organic matter to affect the silver solutions and such should be avoided.

(6) Filter all solutions.

* Bielschowsky, M. *J. f. Physiol. u. Neurol.*, 1904, iii, 169.

† Maresch, R. *Centralbl. f. allg. Pathol. u. path. Anat.*, 1905, xvi, 641.

(7) In our experience equally good results can be obtained by using either yellow or brown chloride of gold.

Of the numerous modifications of Bielschowsky's methods, we are giving but three below. These three would seem to be sufficient for ordinary laboratory work. If further methods are desired, the reader should consult more special works. For laboratories where Zenker fixed tissue and paraffin sections are the rule, we can recommend highly Foot's modification given later (p. 294).

(1) Bielschowsky-Maresch Method. Fixation in 10 per cent formalin is the best. Maresch states that alcohol fixed tissue may also be used but sections from such tissue should be put in 10 per cent formalin for several hours followed by washing in distilled water before being stained. Material fixed in sublimate, osmic acid or chrome salts is not satisfactory, as impregnation is uneven and the silver precipitates out in fine discrete granules.

Frozen sections give the best results but celloidin sections can be used and it is not necessary to remove the celloidin. Paraffin sections may also be used.

Bielschowsky's ammoniacal silver bath used in step (5) is made as follows:

To 10 c.c. of a 10 per cent solution of silver nitrate add first 5 drops of a 40 per cent aqueous solution of sodium hydroxide, then strong ammonia drop by drop, stirring constantly with a glass rod, until the precipitate is just dissolved; an excess of ammonia is to be avoided and this is best done by stopping the addition of the ammonia while there is a granule or two of the precipitate still undissolved. Dilute to 25 c.c. with distilled water and filter. This solution must be made fresh each time before use.

The reduction in step (7) is carried out in a neutral solution of formalin, as the free acid which often occurs in commercial formol interferes with the reducing process.

The gold chloride bath is made up of 10 c.c. of distilled water to which is added 2 drops of a 1 per cent solution of gold chloride and 2 to 3 drops of acetic acid.

The complete procedure follows:

1. Wash formalin fixed tissue several hours in running water before freezing.
2. Cut frozen sections, receiving the sections in distilled water.
3. Place sections for twenty-four hours in a 2 per cent solution of silver nitrate in the dark.
4. Rinse quickly in distilled water.
5. Transfer sections to ammoniacal silver bath for two to thirty minutes depending on their thickness. Here they become yellowish brown.
6. Rinse quickly with distilled water.
7. Reduce in a 20 per cent solution of formalin in tap water for a few minutes up to half an hour, until no more white clouds appear. The preceding rinse in distilled water in step 6 acts as a kind of differentiation for this reducing process. If the rinsing in step 6 is too short, the sections will be too dark; if too long, the sections will not be dark enough; as a general rule, the time should be short.

8. Wash in distilled water.
9. Tone in the acid gold bath until the background becomes red-violet; with sections 10 μ thick, ten minutes usually suffices.
10. Wash in distilled water.
11. Place for fifteen to thirty seconds in a 5 per cent solution of sodium hyposulphite.
12. Wash thoroughly in water.
13. Dehydrate, clear and mount as usual.

By this method, the connective tissue fibers, even the finest, are a deep black on a clear background; thick collagen bundles are more chocolate brown or violet. Nuclei as a rule are not stained to any degree and aniline dyes can be used for staining these.

(2) Perdrau's* Modification of Bielschowsky's Method (as given by Bailey and Hiller).†

Material should be fixed in 10 per cent neutral formalin.

The following special reagents will be needed:

1. Pal's decolorizer—equal parts of a 1 per cent aqueous solution of oxalic acid and a 1 per cent aqueous solution of acid potassium sulphite.
2. Bielschowsky's ammoniacal silver bath, made as follows: To 5 c.c. of a 20 per cent solution of silver nitrate add first, 2 drops of a 40 per cent solution of sodium hydroxide and then strong ammonia drop by drop until the precipitate is just dissolved; dilute to 50 c.c. with distilled water and filter. This reagent must be prepared each time before use.

The procedure is as follows:

1. Wash blocks of formalin fixed tissue twelve to twenty-four hours in running tap water, then twenty-four hours in distilled water, changing the water several times.
2. Cut frozen sections 15 μ to 25 μ in thickness.
3. Wash in distilled water twenty-four hours.
4. Treat sections ten minutes with a 0.25 per cent solution of potassium permanganate.
5. Wash in distilled water.
6. Place in Pal's decolorizer until white.
7. Wash thoroughly overnight in several changes of distilled water.
8. Place in a 2 per cent solution of silver nitrate in the dark for twenty-four hours.
9. Wash in distilled water not more than five minutes.
10. Treat sections forty to sixty minutes with Bielschowsky's ammoniacal silver solution.
11. Wash quickly in distilled water.
12. Reduce thirty minutes in a 20 per cent solution of formalin (not neutral) made with tap water.
13. Wash in distilled water.
14. Tone in a 1:500 solution of gold chloride until sections are an even violet color.
15. Wash in distilled water.
16. Fix in a 5 per cent solution of sodium hyposulphite.
17. Wash thoroughly in distilled water.
18. Dehydrate, clear, and mount as usual.

By this method, the broader bands of collagen are stained reddish and the finer strands, so-called reticulum, black. This method may also be

* Perdrau, J. R. *J. Path. & Bacteriol.*, 1921, xxiv, 117.
† Bailey, P. and Hiller, G. *J. Nerv. & Ment. Dis.*, 1924, lix, 337.

used on paraffin sections if the slides are not agitated in the solutions, otherwise the sections will come off the slide.

(3) Foot's Modification of Bielschowsky's Method. This method was devised especially for Zenker fixed tissue. Formalin fixed tissue may also be used and in our hands has proved quite satisfactory. This method is intended for paraffin sections; we have had no experience with its use on frozen or celloidin sections.

The ammoniacal silver bath used is prepared as follows: To 20 c.c. of a 10 per cent solution of silver nitrate add 20 drops of a 40 per cent solution of sodium hydroxide. The resulting brownish precipitate is dissolved in strong ammonia, which is added slowly, shaking continually; about 2 c.c. will be needed so it is well to add the ammonia drop by drop as this point is neared until the precipitate is almost dissolved. It is better to filter out a few undissolved grains than to run the risk of adding too much ammonia. The resulting solution is made up to 80 c.c. with distilled water and filtered before use. This solution must be made fresh each day as it does not keep.

The solution of formalin used for reduction should be neutral and it is well to change it after the first ten or fifteen minutes.

1. Remove paraffin from the sections in the usual manner.
2. Weak alcoholic iodine solution for five minutes.
3. Weak aqueous solution of sodium hyposulphite until sections are white.
4. Wash in tap water.
5. Treat sections with a 0.25 per cent solution of potassium permanganate for five minutes.
6. Rinse in tap water.
7. Place sections in a 5 per cent solution of oxalic acid for fifteen to thirty minutes.
8. Wash thoroughly in tap water.
9. Rinse in distilled water. Use distilled water for washing until after sections have been treated with formalin.
10. Leave sections for forty-eight hours in a 2 per cent solution of silver nitrate (in subdued light, but not in the dark).
11. Wash a short time in distilled water.
12. Ammoniacal silver solution for thirty minutes.
13. Wash quickly in distilled water.
14. Reduce in a 5 per cent neutral formalin solution for thirty minutes.
15. Rinse at tap.
16. Tone in a 1 per cent solution of gold chloride for one hour.
17. Rinse at tap.
18. Remove excess silver by treating sections with a 5 per cent solution of sodium hyposulphite for two minutes.
19. Wash thoroughly several hours in running tap water.
20. Stain in Harris' hematoxylin ten minutes, or Weigert's iron hematoxylin one minute.
21. Soak in tap water until blue.
22. Counterstain in Van Gieson's picro-acid fuchsin solution for thirty seconds.
23. Dehydrate, clear and mount as usual.

Coarser collagen stains red to rose, finer collagen, so-called reticulum, black to dark violet; nuclei black, blue or brownish; cytoplasm, grayish

yellow; muscle fibers and elastic fibers, more brightly yellow. The finest reticulum fibers are brought out by this method, for if the silver does not penetrate them, the acid fuchsin will, so they are sometimes beaded black on vermilion.

Instead of staining with hematoxylin and Van Gieson's solution, Mallory's acid fuchsin-aniline blue collagen method can be used, especially for the study of fibroglia and myoglia fibrils and for the relation of these to collagen. Sometimes, especially for photographic purposes, it is of advantage to omit the counterstaining with Van Gieson's solution.

(4.) Foot's* Modification of the Hortega Silver Carbonate Method. This method has the advantage over the preceding one because of its saving in time. It can be carried out in less than an hour while the other method requires three days. The results are as satisfactory, if not more so.

The method was devised especially for paraffin sections of Zenker-fixed material.

The silver-ammonium carbonate which must be prepared fresh for each batch of slides is made as follows:

To 10 c.c. of a 10 per cent aqueous solution of silver nitrate, add 10 c.c. of a saturated aqueous solution of lithium carbonate. The resulting heavy white precipitate is allowed to settle and the supernatant fluid is poured off. The precipitate is then washed several times with 25 to 50 c.c. of distilled water, the precipitate being allowed to settle each time and the supernatant fluid poured off. After 25 c.c. of fresh distilled water is added, this washed precipitate is almost dissolved in strong ammonia water, which is added drop by drop while the container is shaken vigorously. About 8 to 15 drops of the ammonia will be needed and great care must be taken not to overstep the end-point of solution. It is better to leave a few grains undissolved than to add too much ammonia. The entire solution is then made up to 100 c.c. with distilled water and heated to 50°C. The slides are immersed in this solution and placed in an incubator at 37.5°C., from ten to fifteen minutes, until they turn a yellowish gray. The temperature of the bath will remain between 40° and 50°C., under these circumstances.

The procedure is as follows:

1. Remove paraffin from sections in the usual manner.
2. Weak alcoholic iodine solution for five minutes.
3. Bleach with 5 per cent aqueous sodium hyposulphite.
4. Wash in tap water.
5. Treat with 0.25 per cent aqueous potassium permanganate solution for five minutes.
6. Rinse with tap water.
7. Leave sections in 5 per cent aqueous oxalic acid solution for ten minutes.
8. Wash well at tap followed with a wash in distilled water.
9. Treat sections for ten to fifteen minutes with silver-ammonium carbonate at about 45°C.
10. Rinse quickly in distilled water.
11. Reduce in 20 per cent neutral formalin for two minutes.
12. Wash well with tap water.
13. Tone in 1:500 aqueous gold chloride solution for two minutes.

* Foot, N. C. *J. Lab. & Clin. Med.*, 1923–1924, ix, 777.
Foot, N. C. and Ménard, B. S. *Arch. Path. & Lab. Med.*, 1927, iv, 211.

14. Wash in tap water.
15. Fix in a 5 per cent aqueous sodium hyposulphite for two minutes.
16. Wash well in tap water.
17. Counterstain, dehydrate and mount as in preceding method.

The staining of the collagen and other elements is the same as in the preceding method.

III. Elastic Fibrils

Three excellent methods are available for staining elastic fibrils. Weigert's is the simplest and most generally useful once the staining solution has been made up. Verhoeff's has the great advantage of staining well after Zenker fixation. Unna's is the oldest but has been largely superseded by the other two.

1. Weigert's* Method for Elastic Fibrils.

(1) Stain paraffin sections twenty minutes to one hour in a fuchsin-resorcin solution prepared as follows:

Fuchsin (basic)	2 gm.
Resorcin	4 gm.
Water	200 c.c.

Boil the solution in a porcelain dish; when it is briskly boiling add 25 c.c. of liquor ferri chloridi (a 29 per cent solution); stir and boil for two to five minutes. A precipitate forms. Cool and filter. The filtrate is thrown away. The precipitate remains on the filter-paper until all the water has drained away or until the precipitate has thoroughly dried. Then return filter and precipitate to the porcelain dish, which should be dry, but which should contain whatever part of the precipitate remained sticking to it. Add 200 c.c. of 95 per cent alcohol, and boil. Stir constantly, and fish out the filter-paper as the precipitate is dissolved off. Cool; filter; add alcohol to make up to 200 c.c. Add 4 c.c. of hydrochloric acid.

(2) Wash in several changes of 95 per cent alcohol followed by absolute.

(3) Clear in xylol and mount in xylol balsam.

For celloidin sections clear from 95 per cent alcohol by the blotting paper xylol method.

2. Hart's† Modification of Weigert's Elastic Tissue Stain.

(1) Stain sections in lithium carmine thirty minutes.

(2) Then directly into

Acid alcohol	100 c.c.
Weigert's stain	5 c.c.

Stain overnight, twelve hours at least.

(3) Wash in 85 per cent alcohol, then dehydrate, clear and mount as in Weigert's method.

3. Verhoeff's‡ Elastic Tissue Method. Fixation in formalin or Zenker's fluid is preferred. Tissues or sections should not be treated with iodine solution before staining. Mercurial precipitates, if removable, are removed

* Weigert, C. *Centralbl. f. allg. Path.*, 1898, ix, 287.

† Hart. *Centralbl. f. allg. Path.*, 1901, xix.

‡ Verhoeff, F. H., *J. Am. Med. Ass.*, 1908, I, 876.

by the staining solution. For the best results the solution should be used within twenty-four hours, but satisfactory specimens may be obtained with solutions one month old.

The staining fluid is made as follows:

Hematoxylin crystals	1 gm.
Absolute alcohol	20 c.c.

Dissolve in test tube by aid of heat, filter, and add in order given:

Aqueous solution (10 per cent) of ferric chloride	8 c.c.
Lugol's solution (iodine, 2; potassium iodide, 4; water, 100)	8 c.c.

(1) Immerse paraffin sections in the staining fluid for fifteen minutes or longer until perfectly black.

(2) Wash in water.

(3) Differentiate in a 2 per cent aqueous solution of ferric chloride. This process requires only a few seconds. To observe the stages in the differentiation, the sections may be examined in water under a low magnification. If the differentiation has been carried too far, the sections may be restained, provided that they have not been treated with alcohol.

(4) Wash in water.

(5) Place in 95 per cent alcohol to remove iodine.

(6) Transfer to water for five minutes or longer.

(7) Counterstain, if desired, in a 0.5 per cent aqueous solution of eosin.

(8) Dehydrate in 95 per cent alcohol followed by absolute.

(9) Xylol, xylol balsam.

By this method elastic tissue is stained black, while connective tissue, fibroglia, myoglia and neuroglia fibrils, myelin and fibrin take the eosin stain. Nuclear staining may be obviated by doubling the amount of Lugol's solution in the staining fluid. Degenerated elastic tissue (elacin) is also stained by this method. The degenerated fibrils may be distinguished from the normal by staining less intensely and presenting less distinct outlines.

Equally good results, especially after Zenker's fixation, may be obtained by staining the tissues en masse. Myelin, however, is also stained. Thin slices of tissue after fixation are removed from 80 per cent alcohol and immersed in the staining fluid four days. They are then quickly rinsed in water to remove excess of stain, placed in 80 per cent alcohol and imbedded in the usual manner. The sections are differentiated in a 0.5 per cent solution of ferric chloride.

4. Unna's* Orcein Method for Elastic Fibers. Unna's latest method of using orcein is as follows, and can be highly recommended:

(1) Stain sections in the following solution:

Orcein	1 gm.
Hydrochloric acid	1 c.c.
Absolute alcohol	100 c.c.

* Unna, P. G. *Monatschr. f. prakt. Dermat.*, 1894, xix, 1.

Place the sections in a dish and pour over them enough of the solution to cover them. Warm gently in an incubator or over a small flame for ten to fifteen minutes until the solution thickens, or leave in the solution at room temperature overnight.

(2) Wash off thoroughly in dilute alcohol (70 per cent).

(3) Wash in water to get rid of all the acid and to fix the color.

(4) Alcohol.

(5) Oil.

(6) Xylol balsam.

The washing in water is not absolutely essential.

Elastic fibers are stained a deep silky-brown color, connective tissue, a pale brown. If it is desirable to have only the elastic fibers stained, wash for a few seconds in a 1 per cent hydrochloric acid alcohol before washing in water. The nuclei can be brought out by staining in Unna's polychrome methylene blue solution after washing the sections in water.

IV. Mucin

Mucin, the homogeneous intercellular substance secreted by the fibroblast under certain conditions, has certain chemical properties in consequence of which it can be stained differentially. It is colored as a rule more or less intensely blue by alum hematoxylin after any fixative and by methylene blue in the eosin or phloxin methylene blue staining method after fixation in Zenker's fluid. The best of the differential stains are the following:

1. **Hoyer's* Thionin Method.** Fix tissues in a concentrated aqueous solution of corrosive sublimate for two to eight hours. Wash and dehydrate in frequent changes of 95 per cent alcohol. Do not remove the mercury precipitate with iodine. Imbed in paraffin in the usual way.

(1) Place sections in a 5 per cent aqueous solution of corrosive sublimate for three to five minutes.

(2) Wash off in alcohol or water.

(3) Stain in a dilute solution of thionin (two drops of a saturated aqueous solution, made by the aid of heat, to each 5 c.c. of water) for five to fifteen minutes.

(4) Wash in 95 per cent alcohol followed by absolute.

(5) Clear in xylol and mount in xylol balsam.

The nuclei are stained blue, mucin, mast cell granules, cartilage and amyloid, red. The mucin appears of a bright red if the sections after step (3) are examined after mounting in water or glycerin. According to Herxheimer toluidin blue can be used in place of thionin and gives more permanent preparations.

2. **Mayer's† Mucihematein Method.** Fix tissues in absolute alcohol, imbed in paraffin.

(1) Stain sections in one of the following solutions for five to ten minutes:

(a) Hematein	0.2 gm.
Aluminum chloride	0.1 gm.
Glycerin	40.0 c.c.
Water	60.0 c.c.

* Hoyer, H. *Arch. f. mikroskop. Anat.*, xxxvi.

† Mayer, P. *Arch. f. mikroskop. Anat.*, xxxvi.

Dissolve the hematein in the glycerin and add the other ingredients:

(b) Hematein	0.2 gm.
Aluminum chloride	0.1 gm.
70 per cent alcohol	70.0 c.c.
Nitric acid	1 to 2 drops.

The first solution stains quicker and better but the alcoholic solution is preferable if the mucin swells much.

(2) Wash in water.

(3) Dehydrate in 95 per cent alcohol followed by absolute.

(4) Clear in xylol and mount in xylol balsam.

Mucin appears blue, the other tissue elements, colorless. The sections can be stained with carmine before using the hematein solution if desired.

3. Mayer's* Mucicarmine Method.

Fixation in absolute alcohol. Paraffin sections.

(1) Stain five to ten minutes in the following solution diluted for use 1 to 10 with water:

Carmine	1 gm.
Aluminum chloride (dry)	0.5 gm.
Water	2.0 c.c.

Heat over a flame for two minutes until the solution appears dark colored. Filter after twenty-four hours. The solution keeps well.

(2) Wash in water, dehydrate in alcohol, clear and mount in xylol balsam.

The mucin is colored red. A preliminary staining with alum hematoxylin is advisable. If the nuclei are stained red it indicates that the staining solution is acid and must be neutralized by the addition of a few drops of a 1 per cent solution of bicarbonate of sodium.

V. Amyloid

Amyloid is an abnormal intercellular product of the fibroblast, chemically related to the ground substance of cartilage. It occurs not only in the liver, spleen and other organs as the result of certain chronic infections and other processes but occasionally in localized masses and rarely in the stroma of tumors. It has definite chemical and physical properties on which certain characteristic staining methods are based.

The original stain for amyloid was iodine which colors it mahogany brown both in gross and in frozen sections. Nowadays more use is made of certain aniline dyes which stain amyloid metachromatically. The great difficulty in the past has been to obtain permanent mounts of sections in which the amyloid was stained characteristically. This has now been rendered possible by Mayer's ingenious and seemingly impossible method. Sections stained several years ago by his method are as bright and sharp today as when originally prepared.

In examining microscopically metachromatic stains for amyloid it is important always to take the light from a white cloud in order to obtain a sharp differentiation in color. The light from the blue sky is worthless.

* Mayer, P. *Mitt. Zool. Stat. Neapel,* 1896, xii.

1. Iodine Reaction for Amyloid.

(1) Stain sections in a weak solution of iodine (Lugol's solution diluted until of a clear yellow color) for three minutes.
(2) Wash in water.
(3) Mount and examine in water or glycerin.

If the tissue reacts strongly alkaline, a condition which may result from post mortem decomposition, the color reaction with iodine will not take place. In such cases the tissue or the sections of it should be treated with dilute acetic acid before applying the test. The normal reaction of amyloid with iodine may be increased by treating the section after staining with dilute acetic acid.

2. Iodine and Sulphuric Acid Reaction.

(1) Stain quickly and lightly in dilute Lugol's solution.
(2) Treat with sulphuric acid, either concentrated or dilute (1 to 5 per cent), on the slip or in the staining dish. Strong hydrochloric acid may be used in the same way.

The color of the amyloid will usually change at once or in a few minutes from red, through violet, to blue. Sometimes the color turns simply to a deeper brown.

3. Langhans'* Method for Obtaining Permanent Mounts with Iodine.

(1) Harden in alcohol and stain in Mayer's alcoholic carmine solution.
(2) Stain sections in Lugol's solution five to ten minutes.
(3) Dehydrate quickly in 1 part of tincture of iodine to 3 or 4 parts of absolute alcohol.
(4) Clear and mount in oil of origanum.

The color is said to keep remarkably well. Other oils or balsam cause it to fade quickly. The staining in Lugol's solution may be omitted, as the tincture of iodine usually stains the amyloid sufficiently deeply.

4. Reaction with Methyl-violet.

(1) Stain frozen sections of fresh or of formaldehyde or alcohol fixed tissue in a 1 per cent aqueous solution of methyl-violet three to five minutes.
(2) Wash in a 1 per cent aqueous solution of acetic acid.
(3) Wash thoroughly in water to remove all trace of acid.
(4) Examine in water or in glycerin.

The stain will keep for some time if mounted in a saturated solution of acetate of potash or in levulose. Other methods are to stain in aniline-methyl-violet and to wash out in a 1 per cent solution of hydrochloric acid, or to stain in a strong solution of methyl-violet to which acetic acid is added, and to wash out in water. The amyloid is stained violet red, the tissue blue. Sections of tissues imbedded in celloidin will not give the reaction unless the celloidin is removed.

* Langhans, *Arch. f. Path. Anat.*, 1891, cxxi.

5. Reaction with Iodine Green.

(1) Stain fresh or hardened sections in a ⅓ per cent aqueous solution of iodine-green for twenty-four hours.
(2) Wash in water.
(3) Mount in water or glycerin.

Amyloid, violet red; tissue, green. Stilling claims that the reaction is surer than with methyl-violet.

6. Reaction with Bismarck Brown and Methyl-violet (Birch-Hirschfeld).*

(1) Stain in a 2 per cent alcoholic solution of Bismarck brown for five minutes.
(2) Wash in absolute alcohol.
(3) Wash in distilled water ten minutes.
(4) Stain in a 2 per cent aqueous solution of methyl-violet five to ten minutes.
(5) Wash in dilute acetic acid solution.
(6) Wash thoroughly in tap water.
(7) Mount in levulose.
Amyloid, red; tissue, brown.

7. Mayer's Stain for Amyloid.

(1) Transfer paraffin sections without previous treatment directly from the knife to a warmed (40°C.) ½ per cent aqueous solution of methyl- or gentian-violet for five to ten minutes.
(2) Wash in water and differentiate in a 1 per cent solution of acetic acid for ten to fifteen minutes.
(3) Wash thoroughly in water.
(4) Transfer to ½ concentrated aqueous solution of alum. Wash off in water.
(5) Transfer sections to slip and allow the water to evaporate.
(6) Remove paraffin and clear with xylol. Mount in xylol balsam.

The same method can unquestionably be used with crystal violet and iodine green.

* Birch and Hirschfeld. Festschr. f. El. Wagner. Leipz., 1887.

METHODS FOR PREPARING MUSCLE AND ELECTRIC ORGAN TISSUES

Ulric Dahlgren

Demonstrating general cellular character of muscle 302. Demonstrating special structural character of muscle 302. Identification of muscle substance by stains 305. Studying cell organs of contraction 306. Study of muscle in any stage of contraction 309. Study of cytoplasmic inclusions 311. Demonstrating cell membranes and sarcolemma and connective tissue attachments 313. Demonstrating other tissue elements associated with muscle cells 314. Study of electric tissues 314.

In considering the procedure necessary to make microscopic sections or other mounts or examinations of muscle tissue or suspected muscle tissue, the following objectives should be taken account of:

1. The general features, as cytoplasm, nucleus, cell wall, centrosomal structures, chromidia, Golgi apparatus, etc., all of which the muscle cell (or syncytium) shares with most other cells. The methods indicated for examining these structures or parts are the same that would be used for similar structures in most other cells, and the worker is referred to the portion on general cytology. These parts are usually overshadowed in our consideration by the cell organs of contraction.

2. General views of muscle.

3. The identification of muscle tissues and cells.

4. The cell organs of contraction, the myofibrils. These are the most important structures in the muscle cell and the most weight will be placed on a discussion of the methods used in studying them.

5. Technique of securing muscle in any stage of contraction or relaxation.

6. The myochondria or muscle fuel substance.

7. The cell membrane and sarcolemma and other outer envelopes of the muscle fiber and the connective tissue attachments and intercellular connectives of muscle cell.

8. Nerve connections or nerve plates, circulatory structures and other foreign structures associated with the muscle cell.

9. The electric cells of fishes (modified muscle cells).

I. Methods for Demonstrating General Cellular Character of Muscle

See technique used in general cytology, Chapter V.

II. Methods for Demonstrating Special Structural Character of Muscle

For this purpose we can use:

(*a*) Teased fresh preparations or total views of small animals such as *Entomostraca*. Such examination is valuable and enables one to use several methods of lighting, as transmitted light, reflected light, dark-field illumi-

nation and polarized light. Some of these, particularly the polarized light, permit very beautiful views of muscle activity and study of the isotropic and anisotropic areas during contraction and relaxation.

(*b*) Sections in paraffin or celloidin of large areas of muscle or body regions in which muscle is found, for the study of the general histology of the tissue and its surroundings. We have to deal here with the subject of muscle fixation, imbedding and staining.

In most cases muscle tissue contains large numbers of myofibrils, and under technical treatment this substance shows a strong tendency to harden and become brittle. Great care should therefore be exercised to employ the proper fixing fluid, as short a fixation as will serve, a rapid dehydration and, in the case of paraffin sections, as short a subjection to heat in the water bath at as low a temperature as possible. Otherwise two familiar artifacts, *pressure ridges** and *section cracks*† will appear in embarrassing profusion.

Paraffin sections can be cut from 1 μ or 2 μ in thickness up to 10 μ or 12 μ. Section cracks are most likely to appear in the thicker sections. Celloidin sections are better for very general views than paraffin sections. Since the tissue does not have to endure the heat of imbedding in paraffin, we are not troubled by the *cracks* and *ridges*. An artifact more apt to occur in celloidin is the condition of *free ends*.‡

1. **Fresh Material.** *a. Teased Tissues.* For striated muscle, bits of vertebrate flesh or arthropod flesh may be used with some success. They should be teased with needles in normal salt or Ringer's solution. In life, under ordinary illumination, this method is not very successful but the individual myofibrils can be seen and the segmentation of each fibril into isotropic and anisotropic substance becomes quite apparent, especially with a strong

* *Pressure Ridges.* In this artifact the knife edge, especially when dull, and encountering a tough substance in a softer paraffin, compresses the material, pushes it slightly forward and then suddenly cuts through it leaving a region of compressed and sometimes darker staining material behind it in the section. This usually leaves a series of such "ridges" in the section which may be misinterpreted.

† *Section Cracks.* This irregularity appears, usually, when a sharp knife edge encounters a hard and brittle substance, well imbedded, and as the section lifts from the block at the angle of the knife edge it cracks at regular intervals, either all the way through or only part way through the thickness of the section. Such cracks are very apparent in the finished and mounted section and their spacing is determined by the distance the knife can go before each crack occurs. In muscle tissue this artifact is much more likely to occur when the fibers are cut longitudinally from one end than when the knife approaches them from the side, while pressure ridges are more frequent when they are cut with the knife-edge parallel with the fiber.

‡ *Free Ends.* This artifact is most likely to occur in longitudinal sections of the muscle fibers. Since the plane of section can hardly ever be made to coincide exactly with the myofibrils, these latter will have many cut ends in the length of the fiber and such ends are very apt to become free at their tips and stick up or lie at an angle to the course of the fibers. This is not difficult to deal with, however, as by focussing slightly down these free ends disappear.

illumination, much cut down by the substage diaphragm. Smooth fibrils (i. e. those without such segmentation) give poor results under such examination, the difference in refractive index between fibril substance and cytoplasm being so small during the life of the cell that the fibrils are seen with difficulty.

b. Whole Animals. A better way to see active muscle is to observe some small living animal, or part of an animal, in which the fibers are so isolated and the surrounding tissues so scarce and transparent that they can be seen through the whole body or member. Pelagic or fresh water *Entomostraca*, certain aquatic insect larvae for striated fibers, and very young small and thin leeches or other *Annelids* or other invertebrate larvae for non-striated fibers, serve this purpose best. Here again the striated muscle is the more easily observed because the difference of refractive index is greater between the isotropic and anisotropic substances of the fibril than between the fibril and its surrounding cell structures.

When polarized light is used, the above examinations of the fibers as a whole becomes much more significant. In this case, as the term implies, the anisotropic and isotropic substances of striated muscle become as sharply differentiated as black from white because the polarized light will penetrate one but not the other. At such times the movements and relations of the two regions in the fiber can be plainly seen or even photographed. Such movements are quick but may be slowed down by cooling.

2. Prepared Materials. *a. Macerated Tissues.* Better visibility under ordinary light is shown by muscle fibers that have been fixed and dissociated and then examined as they are or stained. The fixing process can be made to perform the dissociation as well when fixatives that contain a connective tissue solvent are used. A young trout (3–20 cm. or more), fixed in Bouin's fluid for ten days to three weeks, will yield bits of flesh that can be shaken in a test-tube of water or 70 per cent alcohol so that large numbers of individual fibers will be available. Sublimate-acetic will do the same, not quite so satisfactorily but still adequately. In both cases it is the acetic acid that dissolves the connective tissue. Flemming's fluid and Zenker's fluid are not so satisfactory for this purpose because the chromic compounds work against the acetic acid. Weak osmic acid ($\frac{1}{10}$ of 1 per cent in distilled water) alone will dissociate and fix muscle fibers in an exquisite manner in a few days. With 1 per cent of acetic acid it will dissociate quicker with almost as fine a fixation. In the first case small masses should be used (not over a few millimeters in thickness). In the latter twice that thickness is possible. Ranvier's alcohol ($33\frac{1}{3}$ per cent) will do this work with rather poorer fixation. Smooth muscle from the mammalian bladder is more resistant and from 10 per cent to 20 per cent nitric acid is required for dissociation. This method is applicable for class purposes.

b. Sectioned Material. Frozen sections of striated muscle may be made and observed much as the fresh teased or entire specimens were. Polarized

light and vital stains can be utilized. The method is not so valuable for smooth muscle. The sections are of greatest use when made parallel to the course of the fibers and a little thicker than their diameter. There is no point, in most cases, of using transections of frozen material.

Thicker sections in paraffin or celloidin or the combined method are of great value in giving general views of the superficial structure of both striated and non-striated muscle and in showing the relations of the fibers to the connective tissues, blood vessels, nerve fibers, tracheae, etc., which surround them. For this purpose Zenker's fluid, Bouin's fluid, and many other common fixatives are well fitted. When the connective tissues formed by or associated with the muscle cells are to be a special point, strong acids are to be avoided, especially hydrochloric, nitric and acetic. In these cases pure corrosive sublimate solutions or Zenker and Bouin without the acetic may be used with success. Müller's fluid is most excellent for this purpose when the nuclei and finer details of the cell are not to be considered. This fluid dissolves the chromatin but not the nuclear membrane so that nuclei appear as homogeneous spheres and it sometimes injures the striation. Subsequent staining with Delafield's hematoxylin and eosin should be employed. Iron hematoxylin is not advised with this fixation.

Van Gieson's picric acid and acid fuchsin is very good, showing as it does the muscle substance yellow and connective tissues red, but the first-mentioned stain seems most suited to general work. In general too great reliance on a variety of stains, especially highly complicated special stains, should be discouraged. The continued and repeated use of two or three standard stains with varied timing and concentration may be followed with profit by the use of some of the more special ones. The same may be said of fixatives.

III. The Identification of Muscles Substance by Stains

Striated muscle can be identified at a glance, either stained or unstained, except in very unusual cases when the fixation has been so poor that the striation cannot be seen. On the other hand smooth muscle is often found in positions where its location is unfamiliar or unusual and when it must be distinguished from certain connective tissues. Both act in a tensile connective capacity, both have specific cell organs which are fibrils; in fact one and the same cell may in some cases form myofibrils in one part of its cytoplasm and connective tissue fibrils in another. Myofibrils are always contained in the cytoplasm, though sometimes very near its outer surface. Connective tissue fibrils are often on and outside the surface. When inside, the question often arises as to whether it is a contractile fibril (muscle) or a non-contractile fibril (connective tissue). Such a question still stands in regard to the fibrils in the end cells associated with the luminous organs of the *Lampyridae* and has never yet been answered. To see a fibril contract in life under the microscope is definite and positive proof of its muscular

nature. To see it fail to contract is a dubious negative proof. To find it in one case shorter than in others is an unsatisfying proof. Staining is sometimes the most practical method of making a determination but it should be employed with a certain reserve. When the whole body of small animals or a heterogeneous part of the body of larger animals is sectioned and stained uniformly the method of comparing the questionable fibrils with known muscle tissue is often decisive and is a means often employed.

Most muscle tissues are easily stained with the acid dyes, especially eosin. This alone, however, is not sufficient to distinguish them from many connective tissues; for this reason mixtures have been devised that will stain them differentially. Several such stains should be used with care in making a determination.

1. **Retterer's Stain.** Retterer has devised an excellent and simple stain for differentiating muscle tissue (this is for smooth muscle but it also works well with striated forms) from connective tissues in general. He fixes in 10 volumes of 80 per cent alcohol to which 1 volume of formic acid has been added and then stains in alum carmine. The muscle should show a light red stain while all connective tissues remain unstained.

2. **Van Gieson's Stain.** Van Gieson has formulated a stain for sections, composed of picric acid and acid fuchsin, in which muscle stains yellow and connective tissue red. Others have varied his formula with success which shows that in some cases there must be a greater proportion of the acid fuchsin or the connective tissue will also be yellow, while in other cases there must be a lesser proportion of the fuchsin or both muscle and connective tissue will be red.

3. **Picro-nigrosine.** Picro-nigrosine is a short and rapid stain for distinguishing muscle from connective tissue. Dissolve nigrosine in a saturated solution of picric acid in water. Fixation should be in alcohol or Bouin's fluid. The muscle will be yellow, the connective tissue dark.

4. **Unna's Orcein.** Unna's orcein method after sublimate fixation is good for this purpose with some practice. Stain for twenty-four hours in orcein 1 gm., wasserblau 0.25 gm., alcohol 60 c.c., glycerin 10 c.c., water 30 c.c.; wash in 70 per cent alcohol, dehydrate, clear and mount in balsam. In observing muscle with this stain one must distinguish muscle by comparison with known stains until the mixed purplish color has become familiar. Collagenous and white connective tissues are decidedly blue and elastic connective tissues decidedly red.

The above methods are for use with sections only and for comparisons of the whole muscle cell and its myofibril content of muscle substance with white or elastic or other connective tissue fibrils. (See also **V**, p. 314.)

IV. Methods of Studying the Cell Organs of Contraction

1. **The Myofibrils.** The study of the myofibrils is the most important feature of muscle preparation and investigation. These structures are the

specific cell organs of contraction. They are inside the cytoplasm, are formed and controlled by the cytoplasm and have a higher index of refraction and a greater tensile strength than the cytoplasm. They are always very small, which permits of adequate penetration of reagents and makes shrinkage almost imperceptible.

a. Fixation. The myofibrils are easily fixed by almost any fixative, do not perceptably shrink and tend to stain the same no matter what the fixative. In the different stages of their contractile activity they undergo large chemical changes which are strongly indicated by their staining reactions. It is desirable to study the myofibrils in both longisection and transection and in the former case they should almost always be studied in sections thin enough to contain not more than one or two fibrils between the two planes of the section. Celloidin alone is usually not indicated. Paraffin and the celloidin-paraffin method of double imbedding usually provide the best results. Both a basic and an acid stain should be applied to the specimen in most cases, because the isotropic substance has a strong affinity for the acid stains such as eosin, while the anisotropic material absorbs and is stained by the basic dyes like hematoxylin or safranin. When the two substances are not separated so as to be locally distinguished, the double staining is still valuable to show areas of contraction.

b. Staining. A most important factor of muscle study is to distinguish between *absorption* or *retention* of the dye and *staining* by the dye in the myofibril. Owing to its denser nature the myofibril will absorb or retain in a purely physical way a much larger proportion of the dye than will the cytoplasm; also for the same reason, in striated muscle, the anisotropic substance will retain or absorb much more dye than will the isotropic. Again, the contracted areas of smooth muscle will absorb and retain more stain in this physical manner than the non-contracted. Therefore, in all regressive staining for careful study, the crude stain should be extracted until a real chemical staining remains which will identify the anisotropic substance or contracted area, rather than the absorbed or retained masses of dye that merely indicate its locality. For general class purposes the latter more crude condition is useful and shows very beautiful pictures. In using iron hematoxylin the above should be most carefully considered.

When chemically stained with safranin, gentian violet and other basic aniline dyes, the anisotropic areas and the contracted areas of smooth muscle present stronger pictures than with iron hematoxylin when properly extracted. In seeking to merely locate myofibrils and study their relations to the surrounding cytoplasm the acid dyes, such as eosin, acid fuchsin, etc., should be depended upon.

c. Sectioning. Paraffin sections alone should be used to study the longitudinal sections of the myofibrils in the fiber, while the celloidin-paraffin method will serve in the study of transections of the fibers for observing the relations of the fibrils to the cytoplasm and to each other. This is

because the celloidin prevents the artifact called *tilting** which often occurs with paraffin alone. It is much less likely to occur in very thin sections.

One of the best stains for studying the myofibrils is iron hematoxylin. It should be applied in a variety of ways as has been noted by Heidenhain and many other workers. With striated muscle the stain should first be stopped with only a sufficient decolorization to leave the M or Q stripes, the N stripe and the Z stripe a deep black while the isotropic substance is clear. This is the infiltration stage and is most useful for general views and for most class work.

In another slide the stain should then be extracted until the M stripe is gray, the N stripe has cleared (is usually more bluish) and the Z stripe shows as a sharp black line, in relaxed muscle. If contracted the M stripe will be yellowish and thinner and the Z stripe will be a broad segment in which there is added to the Krause's membrane the substance of adjoining M stripes which have obliterated the N stripe and masked the Z stripe (Krause's membrane) so that the dark bands of the muscle appear to have been moved from the M position to the Z position leaving the M position clear and unstained, save for a dim line in some forms. In excessive contraction the Z bands have been observed to be one-eighteenth the length of the resting units and so closely approximated that they touched, thus making the substance an apparently almost solid mass. In certain post-contractile stages the staining power appears to be lost, leaving only one band present, the Z line. We will omit here the various intermediate stages and appearances which belong more to a discussion of muscle histology than to an exposition of technique.

Some of the other valuable stains for the striated myofibrils are Mallory's phosphotungstic acid hematoxylin and Mallory's aniline blue connective tissue stain after fixation in Zenker's fluid (p. 421).

In smooth muscle myofibrils are also present. They respond on the whole to fixatives and stains much as the myofibrils of striated muscle do. Zenker's and Flemming's strong mixture are among the best fixatives. Iron hematoxylin stands first as a stain. The fibrils are not divided into contractile units (sarcous elements) as in striated fibers, but contract in irregularly placed regions called by McGill "contraction nodes" in which the fibrils shorten and thicken and change in their chemical make up so that they are evidently denser and retain dyes both by infiltration (or absorption) and by chemical union (staining).

2. Krause's Membrane. This membrane divides the sarcous elements (contractile units) of striated muscle from each other. It is of a higher refrac-

* A fourth artifact may be known by the name of *Tilting*. This occurs only in transections of muscle fibers and is very common there. In an exact transection the myofibrils should point exactly up toward the observer. When tilting occurs some or all of them have tipped over so that an oblique or even lateral view is presented. This artifact takes place in paraffin sections much oftener than in celloidin or paraffin-celloidin sections or regular celloidin sections and in thick sections oftener than in thin ones.

tive index than any other cytoplasmic element in the muscle cell and takes the black of iron hematoxylin strongly. The myofibrils pass at right angles through it and the membrane extends out into the free cytoplasm from side to side of the fiber where it joins with the sarcolemma. It may be stained at times by Bielschowsky's silver stain. It is non-contractile but may be expanded and made wavy by fixation with boiling water. When the muscle contracts its non-extensile character causes the cytoplasm to bulge out between the points of attachment. By treatment with 98 per cent alcohol the muscle fiber becomes brittle and tends to break all the myofibrils at the intersection with Krause's membrane into a number of transverse discs. Weak chromic acid, on the contrary, may be used to rupture Krause's membrane between the myofibrils and thus free them into longitudinal elements.

The N stripe during relaxation is stained bluish gray by iron hematoxylin. It may also be demonstrated by the Golgi silver stain. This structure lies in the cytoplasm and is not a part of the myofibrils but is mentioned here because it is very often erroneously considered to be a secondary anisotropic region of the myofibrils, which it is not. During contraction the stainability and increased density of this region cause this structure to become invisible.

V. Method for the Study of Muscle in Any Stage of Contraction

1. Striated Muscle. To secure any definite stage of contraction in a muscle preparation has proved a more difficult thing than might be thought. Though an animal be dead the muscle tissue may still show reaction to stimulation. The mode of tissue death often determines the physiological picture presented by the stained slide. The length of the muscle when tissue death occurs does not always establish the physiological stage of contraction shown, as a myofibril in such stage may be stretched and still retain the picture of physiological contraction. Ordinary preparations, where the tissue from an animal killed by decapitation is thrown into the fixative, will show in nearly all cases a stage of weak contraction which is sometimes varied by occasional areas of stronger contraction.

One of the best animals from which to secure striated muscle in all stages of contraction is the large larva of *Corydalis cornutis* which can be found in quantities under the larger flat stones of most of our medium-sized running streams. These animals are tough but soft, and very muscular. The skin can be very easily cut by the microtome knife and does not have to be removed.

One animal should be chloroformed until entirely lax and dead. It is then pinned out straight on a cork sheet and its body cavity filled with Flemming's or Bouin's fluid by injection with a hypodermic syringe. After five to ten minutes, with a sharp pair of scissors cut along each side of the body so that the upper and lower body walls can be readily separated, the internal organs and fat-bodies removed and the two principal muscle

masses further fixed and treated for sectioning. When cut and stained such muscle should show very little contraction. Fixation with boiling water followed by Bouin's will also show very clear and bright pictures but the z line will be slightly swollen and waved.

FIG. 1. Longitudinal section of a bit of the body muscle of *Corydalis cornutus*. Relaxed state. Fixed in Bouin's fluid. Stained in iron hematoxylin. Fixed by body injection of fixing fluid. Prepared and photographed by C. S. Shoup.

Pin out another live animal in the same way and hook the copper wire poles of 2 dry cells of 1½ volts each with an inductorium in the circuit. Draw out the induction coil to between 3 and 4 cm. so that a tetanic current will pass through the animal's body when the current is turned on. Then insert the hypodermic needle into the body cavity and, just after the current connection is made, flood the body cavity with Flemming's or Bouin's fluid, keep the electric current running for about thirty seconds and then cut the animal open as before and complete the fixation and hardening, etc., and cut sections.

Muscle should be found in such sections in all stages of contraction. Some muscles with contraction waves will be found in which the process can be followed from inception to finish; also fatigued muscle that has relaxed. In the contracted portions, sarcous elements will appear that have contracted to $\frac{1}{18}$ of the length of those in resting fibrils (Figs. 2 and 3). This is more than a normal contraction and in consequence some fibers will be found ruptured. Another animal should be treated in the same way with a weaker current for comparison.

2. Smooth Muscle. Smooth muscle may be secured in different stages of contraction and showing contraction waves or areas by simpler means. A bit of freshly killed cat's intestine is cut out and exposed to the cooler

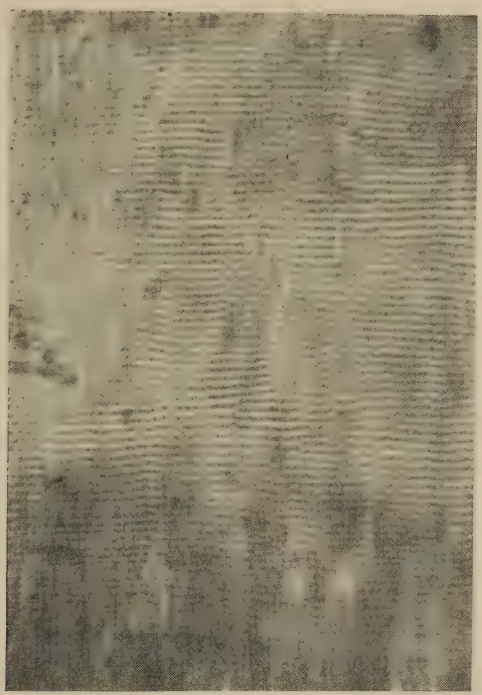

FIG. 2. Longitudinal section of a bit of the body muscle of *Corydalis cornutus*. Excessive contraction under a three volt current from an inductorium. Coil extended 4 cm. Same scale as Figure 1. Same fixation and stain as Figure 1. Prepared and photographed by C. S. Shoup.

air of the room or rubbed with the handle of a scalpel. Sections will show many contraction areas. Or the animal may be chloroformed to death and, just as rigor mortis is setting in, bits of bladder or intestine quickly fixed will show fully extended smooth fibers.

The foot and valve muscles of marine *Pelecypod* mollusks may be completely relaxed by an excess of calcium salts in the sea water. This same muscle, freshly cut out, will be contracted. Other methods remain to be devised for these and other animals, especially some of the invertebrate forms.

VI. Methods for the Study of Cytoplasmic Inclusions

The cytoplasm of muscle cells contains in normal tissues a large but varying amount of material which has been stored there by its metabolic activity against the needs of its contractile activities. This material is

usually in a fluid condition but in some muscles it may appear as a solid. When visible, it ranges in appearance from a reticulated precipitate to small granules and up to large granules with a definite form and location.

FIG. 3. Longitudinal section of a bit of the body muscle of *Corydalis cornutus*. Just relaxed after contraction under electric stimulation. Same fixation and stain as Figure 1. Same scale as Figure 1. Prepared and photographed by C. S. Shoup.

The best place to see them is in the thoracic wing muscles of the solitary hornet *Sphecius*, and probably in other allied forms. In *Hydrophilus* these bodies, which may be termed myochondria, are large and visible but not arranged regularly. We have no differential stain as yet to identify this material but when in granules it can be adequately located by fixing the muscle in sublimate acetic or chromacetic-formalin, cutting thin sections and staining with iron hematoxylin. The sections should be not more than 5μ thick and the iron hematoxylin should be withdrawn until the M stripe is decolorized and only a faint image of the Z line can be seen in the large myofibrils. At this point the myochondria will remain a deep black with absorbed hematoxylin and the decolorizer should be quickly washed out. A counterstain such as eosin, may or may not be valuable.

The regular arrangement of the myochondria in *Sphecius* is so marked, two between each adjoining pair of sarcous elements, that most observers are easily deceived into believing that they are looking at the black M

stripes when they are only looking at the transverse rows of myochondria in the cytoplasm between the myofibrils. The best way to see the difference easily is to observe the thinned edge of a wedge-shaped section which often occurs when the paraffin block is a little too large and hard. Here the loose end artifact displaces some of the fibrils in longitudinal sections. Also in thin transections the arrangement becomes plainer.

In the heart muscle of *Homarus* certain spike-shaped or tack-shaped bodies in the cytoplasm are rendered visible by several stains, the best of which is Delafield's hematoxylin.

In most muscle fibers the cement substance that binds the myofibrils into groups has not been differentially stained except as to degree.

VII. Methods for Demonstrating Cell Membranes and Sarcolemma and Connective Tissue Attachments

A sarcolemma or thin outer covering of connective tissue has been demonstrated in all larger muscle cells. This ranges from a covering of thin connective tissue secreted from regular connective tissue cells that are in the neighborhood, through membranes partial or complete, secreted by the muscle cell's own cytoplasm, to muscle cells in masses where this outer cytoplasmic activity is confined to the formation of strands of connective substance which serve to bind spindle-shaped cells together and to take part of the contraction strain that may come on them. The technique of demonstrating these outer coverings is varied and has developed from numerous scientific disputes as to whether a sarcolemma was present or not, and if so whether it was secreted from the muscle cell or from surrounding connective tissue cells. The following techniques have been devised to meet these cases, in which the writer does not enter into the merits of the disputes as to the origin of the structures involved:

1. **Striated Muscle.** To demonstrate the sarcolemma of striated muscle in vertebrates Ranvier throws a live frog into boiling water. Muscle fibers in the thighs are ruptured and the ends are retracted leaving the sarcolemma intact and passing between the ruptured ends where it is visible in the unstained specimens teased out in salt solution. Solger demonstrates the same structure by teasing fresh striated muscle in a saturated solution of ammonium carbonate under the microscope. Thin transections of muscle stained in iron hematoxylin and eosin, after Zenker and Flemming's fixation, show the thin line of the sarcolemma taking the eosin, if sufficiently decolorized after the hematoxylin, but retaining a gray to black color if still infiltrated with the hematoxylin.

2. **Smooth Muscle.** In smooth muscle and all muscle masses containing spindle-shaped cells, the connective tissue fibrils that unite the cells of the mass are secreted by the ectoplasm of the muscle cell itself. These fibrils are flat and form a reticulum in the smooth muscle of mammals. They may best be seen in sections of the bladder wall hardened for seven

to ten days in Flemming's strong mixture and stained in iron hematoxylin, a long stain (twelve to sixteen hours) with a decolorization just sufficient to leave the muscle cells clear yellow, when the connective tissue fibrils will appear from dark gray to black. Bielschowsky's silver stain will, in the majority of cases, show the muscle cells light yellow (unstained) and the connective tissues fibrils light to dark brown. Van Gieson's picric acid fuchsin stains the connective fibrils light pink, and is unsuitable owing to their delicate nature. An especially good demonstration of such connective tissue fibrils is to be seen in transection of the aorta or other large arteries leaving the heart of cephalopod mollusks. Here the blunt ends of the striated circular muscle cells show these connective tissue fibrils radiating to points of attachment on the sides of the adjoining muscle fibers and only an iron hematoxylin stain is necessary to demonstrate them.

Where muscle fibers are attached to bone, cartilage, shell etc., it is sometimes difficult to decide where the myofibril of the muscle fibers ends and an intervening connective fibril begins. Usually an epithelial cell (mollusk, insect etc.) or a connective tissue cell (vertebrate) intervenes and the myofibrils appear to be continuous with connective tissue fibrils belonging to these two kinds of cells. A chemical differentiation may best be shown in these cases by Mallory's aniline blue stain (p. 308).

In addition to these white connective tissue fibrils and reticular structures a network of very fine elastic fibrils is found in some muscle masses, running around and between the muscle cells. To see these, use some good elastic tissue stain such as Unna's methylene blue and acid fuchsin after fixation with Flemming's strong mixture. Stain sections five to seven minutes in polychrome methylene blue, wash in water and then stain them for ten to fifteen minutes in an 0.5 per cent solution of acid fuchsin plus 33 per cent of Grübler's tannin mixture. Elastic fibrils should be the only blue fibrils in this preparation.

The heart muscle of vertebrates shows at regular intervals in the fibers a series of lines of cell attachment, the intercalated disks or cement lines. Such a line occupies a position in the striation corresponding to the location of Krause's membrane, from which it may be derived, and is best stained with Mallory's phosphotungstic hematoxylin after a Zenker fixation. It is not easily stained and may not be clearly visible in all parts of the section.

VIII. Methods for Demonstrating Other Tissue Elements Associated with Muscle Cells

For this technique see page 356.

IX. Methods for the Study of Electric Tissues

These tissues, known in only seven types of fishes, are derived from muscle cells and one characteristic is that they retain in varying degrees the firm, easily fixed myofibril masses found in muscle. In the more highly specialized electric organs the electroplaxes have least of this substance

and are more difficult to secure good preparations from, shrinkage being the factor to be guarded against. The cytoplasm, in which such myofibrils as are present lie, is peculiarly soft and contains much water-soluble material that passes out during the technical processes. Consequently the tissues may be divided into two groups, those with the larger amount of residual myofibrillation, enough to make successful preparations probable, as *Astroscopus*, *Raja* and *Mormyrus*, and those which show but little or no residual myofibrillation, as *Malopterurus*, *Electrophorus*, *Torpedo*, and *Gymnarchus*. The following two methods of fixation and imbedding have been found to be the only ones so far that will preserve the general form of the units of the second group in anything like their natural form and size. For these one of the following two methods should be used:

(1) Place bits of the tissue, a centimeter in thickness, in Flemming's strong mixture and allow them to remain for three or more weeks with one change after about twenty-four hours. Wash in running tap water for six hours and run up slowly in the alcohols. Imbed carefully in celloidin, first a thin solution and then the strong mixture. Harden the block in pure chloroform until dense, change to a mixture of two parts chloroform and one of cedar oil and cut sections as thin as convenient. Stain with iron hematoxylin and mount. Or, with but slight further shrinkage, the block of celloidin may be cut off the carrier and imbedded in paraffin and thinner sections cut, floated out on warm water on the slip, cooled and dried fast to it and an almost equally unshrunken picture secured. These latter sections can also be stained by safranin and any other dyes that can be used after Flemming's mixture.

(2) This method depends on formalin. Bits of the tissue may be fixed either in 10 per cent formalin, or in any fixative like sublimate or picric acid containing this amount of formalin, for three or more days and then imbedded and cut as above in celloidin. This will permit of other staining but the double imbedded specimens will suffer somewhat more shrinkage than the Flemming's material.

These two methods must be followed in the second group and may be followed in the first group of electric tissues with advantage. All other ordinary fixing methods may be employed with the first group but the imbedding must be done with care. Owing to the presence of a semi-permeable membrane on all electroplaxes osmotic action is strong and especially in the case of the skate (*Raja*) great distortion produced by this condition is common. Often in electric tissue from this fish the membrane which covers the entire electroplax will swell up like a balloon with the cytoplasm and myoid substance sticking to the anterior or the posterior curvature.

No specific stains have been discovered for electric tissue. The myoid substance stains much like muscle, showing only a little less affinity for eosin. The large nerve connections are easily stained with nitrate of silver by Bielschowsky's or Golgi's silver methods, perhaps more easily than those found in muscle. The electric connective tissue found between the electroplaxes stains much like lax white connective tissue. Embryonic electric tissues act much as do other embryonic tissues. The peculiar soft-

ness and soluble contents of the electric elements appear at an early stage, however.

The principal cytoplasmic inclusions of the electroplax, as in muscle, seem to be an energy-storing substance that is most often fluid but in some cases assumes the form of solid granules. This substance is solidified by the osmic acid in Flemming's fluid and by formalin treatment, as is implied above. When overtreated with Flemming's strong mixture it becomes blackened, so that one should, in using such a section, refer to those electroplaxes that lie just inside the more heavily blackened layers. The central portions are often useless because the substance has been dissolved out in the preparation. Iron hematoxylin or safranin are good stains.

In the *Torpedo* electric organ that has been carefully prepared by even ordinary methods and cut in paraffin the thickness of the electroplaxes is often less than the diameter of the nuclei, which then bulge out the upper and lower membranes. In carelessly prepared specimens so sectioned the upper and lower membranes are all that is left and one must be an expert to say whether any given nucleus is within one or between two electroplaxes.

Much work has been done on electric tissues by special methods. They are easily dissociated and mounted whole to advantage by any dissociation methods. To see the myo-striation in *Astroscopus*, *Raja* and *Mormyrus*, dissociate in 15 per cent nitric acid and mount unstained in glycerine-jelly. The nerve endings have been successfully studied by the silver methods of Golgi and Bielschowsky. Also with methylene blue by Retzius. Electrochondria or the solid form of the stored fuel material has been demonstrated in *Mormyrus* and *Malopterurus* by staining with iron hematoxylin after Flemming's fixation. These granules, however, appear as almost unstained or yellowish bodies in the cytoplasm. The "rods" in the electroplax are stained black by absorbed iron hematoxylin and gray by further extraction (the true stain). They may be differentially stained clear red on a purplish ground by acid fuchsin in Mallory's connective tissue stain as shown by Hughes.

NEUROLOGICAL TECHNIQUE

W. H. F. Addison

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Introduction

The chief methods devised for the study of special features in the nervous tissues are outlined in this chapter, as well as some of the general methods which have been found by experience to be useful in this field.

In the accompanying tabulation these methods are arranged under several headings, the central and peripheral subdivisions of the nervous

Table 1

NEUROLOGICAL METHODS

Central nervous system	I.	Removal of nervous tissue from body.		
		Isolation of nerve-cells.		Dissociating fluids
		General anatomical structure.		Many methods, e. g., 10 per cent formalin, hematoxylin and acid fuchsin
		Cell size and growth changes.		Bouin's fluid; methylene blue, thionin, etc.
	II. Special structures	Cell-bodies	Nissl bodies	Basic aniline dyes, such as methylene blue, thionin, toluidin blue, cresyl violet
			Neurofibrils	Methods of Cajal and Bielschowsky
			Internal reticular apparatus	Cajal's uranium-formalin method
			Mitochondria	Regaud-Kull method
			Glycogen	Gage's method
			Trophospongium of Holmgren	
			Centrosomes	
			Pigment	Rio-Hortega, Hatai
		Processes	Myelinated	Methods of Weigert, Pal-Weigert, iron hematoxylin of Heidenhain
			Unmyelinated	Methods of Cajal and Bielschowsky
		Cell-bodies and processes together		Impregnation methods of Golgi, Cox-Golgi, Cajal, Bielschowsky, vom Rath
		Degeneration in cells, chromatolysis		Methods for Nissl substance
		Degeneration in myelin sheaths		Method of Marchi. Also fat stains, e. g., Sudan III
		Neuroglia		(See special chapter)

TABLE II

Peripheral nervous system	I.	Isolation of nerve-fibers		
		General anatomical structure		All general fixatives, as 10 per cent formalin, Bouin's fluid, Zenker's etc.; hematoxylin with eosin or with acid fuchsin or van Gieson's
		Distribution of nerve components in small animals and young stages		10 per cent formalin, followed by vom Rath's method (Elasmobranchs)
				Cajal methods for calcified tissues, and Huber and Guild's modification (Mammals)
		Size and growth of myelinated fibers		Osmic acid, 1 per cent
	II. Special structures	Cell-bodies of cerebrospinal and sympathetic ganglia		As in central nervous system
		Processes	Myelinated	Osmic acid, methylene blue intra vitam
			Unmyelinated	Ranson's pyridine silver, Ranvier's gold chloride, methylene blue intra vitam, Cajal, Bielschowsky
		Cell-bodies and processes together		Ranson, Cajal, Bielschowsky, methylene blue intra vitam
		Degeneration	In cells, chromatolysis	Methods for Nissl substance
			In myelin	Marchi, Sudan III
			In unmyelinated fibers	Ranson's method
		Nerve endings		Methylene blue intra vitam; Ranvier's gold chloride; Cajal methods; and modifications for calcified tissue; Huber and Guild; Bielschowsky; Sihler-Gad

system and their structural elements. This may facilitate the selection of suitable methods for the desired purpose.

In no field of technique, perhaps, are minor modifications so necessary and therefore so numerous as in this one. Weigert, who early worked out one of the most important procedures, was rather nonplussed by the number of modifiers of his method. But he himself made several modifications, and experience has shown that many different factors, such as species of animal, age, myelination and functional condition, as well as other imperfectly known factors, demand a variation in the procedure. The result is that one has to rely on the experience gained by repeated trials.

If only a general survey of the tissue is desired, certain general histological methods are useful, but if a special study of relationship of neurons, growth, or cytologic details, then special appropriate methods are demanded.

I. Removal of Nervous Tissues from Body

In the preparation of the nervous tissues for study the first step is the careful removal of the material from the body into the fixing fluid. In the case of small embryos the whole specimen or a part of it, such as the head, is usually prepared entire. In large fetuses or the young of small animals the top and sides of the brain are exposed by removal of the cranium but the brain may be allowed to remain in the base of the cranium until after fixation and washing. In larger animals the brain is usually removed by freeing it on all sides. This is an important step in securing good results. The general rule is not to handle, press on, or stretch the part desired, but to dissect and cut away surrounding structures until the part is exposed.

In the removal of the central nervous system, one needs bone forceps of several sizes, and sometimes fine-toothed saws, in addition to the usual dissecting instruments. In taking out the human brain the ordinary post-mortem technique is followed. In the case of smaller animals, smaller sizes of instruments must be used. Thus in removing the brain of an albino rat, it is advisable to use manicure forceps, and fine-pointed scissors. After complete evisceration, or thorough bleeding of the animal, the skin of the head and the muscles of the occipital region are cleared away to the bone. One point of the fine bone forceps is inserted gently between the atlas and one of the occipital condyles, keeping the inner point of the forceps close to the latter, and with pressure the condyle is broken. Then the other condyle is similarly cut. The occipital bone can then be turned upward and the dorsal surface of the medulla and part of the cerebellum exposed. It is usually advantageous also to cut the atlas on both sides and remove the upper part thus separated. Then by cutting forward with the bone forceps on each side, the lateral and dorsal aspects of the cranium are quickly taken off in small pieces. In young animals the bones readily separate along their sutures. The chief difficulties are with the paraflocculars lobes of the cerebellum and with the olfactory bulbs. Each of the former lies in a lateral bony recess which has a narrowed connection with the cranial cavity. The dorsal margin of this recess must be carefully chipped away to free the paraflocculus. The olfactory bulbs at the nasal end of the brain are held closely to the bony floor by the olfactory nerves which traverse the openings in the cribriform plate, and it is best to cut these nerves last of all. The membranes over and between the two bulbs should be pulled up gently, and removed so that the margins of the bulbs are entirely free.

The brain is removed from the cranium by holding the head of the animal with its nose pointed downward and with fine scissors cutting, at the tip of the calamus scriptorius, the junction of medulla with spinal cord and then the most posterior cranial nerves. As the weight of the medulla, aided by gentle pressure with the end of the forceps, causes it to fall away from the bone when these nerves are cut, the more anterior nerves come into view and are cut through in succession with fine-pointed scissors.

Finally only the olfactory nerves remain, and these are cut across, and the brain allowed to drop into the dish of fixing fluid and to rest on the layer of absorbent cotton which covers the bottom of the dish.

If only a small portion of the brain is to be taken the region around the part desired should be well exposed. Then with a safety razor blade or thin-bladed knife, cuts should be made completely through the brain on both sides of the part to be taken. Remove one of the adjoining segments, cut any nerves or membranes holding the part desired and then lift this on a spatula of appropriate size into the fixing fluid, or let the tissue fall into the fluid directly.

When removing peripheral nerves, these should not be stretched or pressed with forceps. After the muscles have been removed or separated to expose the nerve a piece of paper is laid on the part desired and the nerve cut across at both ends. By raising first one end of the paper cautiously, the moist nerve adheres to it, and the connective tissue fibers below the nerve can be cut with scissors and the nerve liberated from its surroundings. The paper is then placed with the nerve downward, on the surface of the fixing fluid. In the case of nerve trunks of large animals or of human nerve trunks, a glass rod or stiff bristle may be laid alongside the nerve and the nerve tied with soft string to the ends of the rod. Then the nerve is cut beyond the ligatures. The glass rod and the attached nerve are then stood up in the bottle of fixing fluid, so that the nerve is practically surrounded by the fixing fluid, and is not in contact with the inside of the bottle.

II. Isolation of Nerve Cells

A rapid but crude method of viewing nerve cells with considerable lengths of their processes attached is to make a film preparation, as in examining blood. A small piece (1 to 2 mm. across) of gray matter of the ventral horn of fresh spinal cord is squeezed flat between two cover slips, and these are then slid apart. The film dries quickly in air, and also may be dipped in 95 per cent alcohol for a few seconds and allowed to dry. The films are then stained with 1 per cent aqueous methylene blue or other basic aniline dye, for two to three minutes, washed in water, dried and mounted.

A more careful method is to place small pieces of fresh gray matter in a dissociating fluid, as Ranvier's ⅓ alcohol, or Gage's formaldehyde dissociator. The latter is made of formalin (full strength) 2 c.c., with normal physiological solution 1000 c.c., and should act for two to three days. Large cells, as the multipolar cells of the ventral horn, may be dissected out under the binocular microscope. When the cells are partially freed by careful teasing, the pieces of tissue should be stained, as by carmine or picrocarmine, or by a dilute aniline dye. Excellent preparations are often to be obtained in this way.

III. Methods to Show General Anatomical Structure

1. Formalin 10 per cent, Bouin's Fluid, Orth's (Formol-Müller) Fluid, Formalin and Alcohol Mixtures. Since the introduction of formaldehyde solution as a preservative in 1893, it has largely superseded Müller's fluid as a general fixing agent for the nervous system. Formaldehyde is a gas, soluble in water, and the concentrated solution is sold as formalin, formol, etc. It will be referred to here as formalin. Human brains are generally preserved entire in 10 per cent formalin (i. e., 10 parts of the commercial product with 90 parts of water) and may be kept in it for years without change in outer form (but with a certain increase in size and some loss of solids). Formalin and its dilutions are usually acid from the presence of free formic acid, but they may readily be neutralized by having a layer of magnesium carbonate or several pieces of marble on the bottom of the container. Formalin solutions penetrate rapidly and give the tissue a firm consistency. If necessary, thin slices of fresh brain tissue may be immersed in it heated to near the boiling point, for a minute, as a preliminary to cutting frozen sections for quick examination; but such treatment is not advised. After formalin fixation the material may be imbedded in paraffin or parlodion, and the sections stained with 1 per cent aqueous methylene blue, hematoxylin and eosin or acid fuchsin, etc., in the usual manner.

Formalin also serves as the fixing agent in several special procedures: as the myelin stain method of Weigert, the silver impregnation method of Bielschowsky, and some of the modifications of the reduced silver method of Cajal.

Other fixing fluids suitable for preserving whole brains or large portions of them, are Bouin's, Orth's, and Müller's fluids. They are used as for other tissues, and histological sections from material thus preserved are stainable by all the routine methods.

Hrdlička* experimented with mixtures of formalin and alcohol, and found certain ones which produced only slight initial changes in weight, followed by practically no further change. The proportions should be altered in accordance with the total weight of the brain.

2. Hrdlička's Formulas, for Preserving Brains, Human and Comparative.

	Distilled Water Parts	95 Per Cent Alcohol Parts	Formalin Parts
Brains up to 50 gm. in weight	45	52	3
Brains 51 to 150 gm. in weight	40	57	3
151 to 300 gm. in weight	35	62	3
301 to 900 gm. in weight	30	67	3
above 900 gm. in weight	25	75	3

* Hrdlička, A. *Proc. U. S. Nat. Mus.*, 1906, xxx, 245.

Quantity of fluid: for all specimens above 30 gm. in weight use 4 c.c. to the gram; for brains 15 to 29 gm. use 6 c.c. to the gram; for brains less than 15 gm. use 75 c.c. per specimen.

For brains of fetuses and the very young it is well to use 10 per cent formalin made up with a half-saturated solution of alum in water, in order to increase the hardening.

By adding to 5 per cent formalin solution enough sodium chloride to make the specific gravity 1.030 (about that of the human brain) the initial change was much less and was usually a decrease in weight. This method was originally suggested by Spitzka for the conservation of human brains. As the brains practically float, just submerged, in the mixture, there is no distortion due to pressure on the sides of the container, and this is highly desirable in preserving human brains, whether intended for dissection or for microscopic study.

IV. Methods to Show Cell Size and Growth Changes

Bouin's fluid has been largely used for these purposes on mammalian tissues in recent years. In the study of growth of layers, as of the cerebral cortex, or of cells, as of Purkinje cells, it is necessary to select as fixing fluid one which produces as little deviation as possible from the size in the living condition. This aim is not easily achieved. Immersion of nervous tissues in some fixatives is followed by swelling, in others by shrinking.

Several factors, in addition to the fixing fluid itself, have a considerable influence on the final size of the brain. These are freshness of the brain, presence or absence of soft membranes, age, initial size of brain, amount of fluid used and temperature of the air.

Donaldson* using sheep brains, found nearly 40 per cent increase in weight after fixation in 2½ per cent potassium dichromate, and nearly 40 per cent decrease in weight after the use of 95 per cent alcohol.

Plant† studying the factors influencing the behavior of the brain of the albino rat in Müller's fluid, found a rapid swelling for a week and then a steady loss until the seventy-fifth-day weighing, when the brain still weighed 20 to 30 per cent more than the fresh weight. The factors to which this range in amount of swelling (20 to 30 per cent) was due were chiefly: age of animal, initial brain size, and percentage of water in the brain. It is evident that fallacious results might follow from the use of these fixing fluids in growth studies.

Hrdlička (p. 321), using both human and animal brains, studied the effects of formalin solutions, and found a sharp initial rise in the weight of the specimens, reaching a maximum within less than a week, with a subsequent gradual, long-continued loss. On sheep brains 10 per cent formalin caused an increase in weight of 15 per cent by the end of the first week,

* Donaldson, H. H. *J. Morpbol.*, 1894, ix, 123.

† Plant, J. S. *J. Comp. Neurol.*, 1919, xxx, 411.

which was reduced to 10 per cent at the end of the second month. This gradual loss is attributed to the dissolving of certain brain constituents by the watery fluid.

H. D. King* studying the effects of 10 per cent formalin on brains of albino rats, found that it produced a pronounced swelling at all ages, and that a 10 per cent solution of formalin, neutralized with sodium carbonate, produced a much greater swelling than does a solution with a faintly acid reaction. Also 10 per cent formalin, acting for periods of one month and over, extracts solids from brains of all ages. Brains of very young rats (from birth to ten days) lose 30 per cent of their solids, while brains of adults lose 2 per cent.

Percentage increase of weight of brains, 3 ages, 2 fixation periods:

	Age		
	10 Days Per Cent	100 Days Per Cent	200 Days Per Cent
Three-day period of fixation.....	52.1	36.9	33.1
Four-week period of fixation.....	41.1	25.3	21.9

In these reactions of the brain substance to various fixatives, the high percentage of water in nervous tissues is an important factor. Thus Donaldson† found that in the brain of the albino rat at birth the percentage of water was 88 per cent, and at one year 78 per cent; in the spinal cord, 88 per cent and 70 per cent respectively. In the neurons the percentage of water is 87 per cent throughout, and in the myelin sheaths 48 per cent. The decrease in the percentage of water in the brain and spinal cord with increasing age is intimately connected with the process of myelination, and therefore especially affects the white matter. In man in the gray matter of the cerebral cortex the percentage of water is 88 per cent at birth and 86 per cent at maturity, while in the white matter (corpus callosum) the percentage is 88 per cent at birth and 70.4 per cent at maturity.

The quantitative chemical changes in the solid substances of the human brain during growth are given by Macarthur and Doisy.‡

By histological examination of brains fixed in 10 per cent formalin, however, King§ showed that this substance did not have as injurious an effect on the structure of the cells as did other fixatives that produced much less alteration in brain weight. After fixation in 10 per cent formalin and imbedding in celloidin, there was no apparent shrinkage of the cell-body, the cytoplasm stained evenly and appeared uniformly distributed. The nucleus, however, was decidedly larger than normal, its reticulum poorly preserved and faintly staining. Of the many combinations of formaldehyde which King tried, the picro-formol-acetic mixture of Bouin was the one

* King, H. D. *J. Comp. Neurol.*, 1913, xxiii, 283.

† Donaldson, H. H. *J. Comp. Neurol.*, 1916, xxvi, 443.

‡ Macarthur, C. G. and Doisy, E. A. *J. Comp. Neurol.*, 1919, xxx, 445.

§ King, H. D. *Anat. Record*, 1910, iv, 213.

which produced practically no alteration in brain weight, and besides gave an excellent preservation of the nerve-cells.

Holt* studying the volume of the olfactory bulbs in the albino rat, examined the changes in volume caused by Müller's, Orth's, and Ohlmacher's modification of Carnoy's fluid. Of the three, Müller's caused the greatest increase, and the final weight was reached at periods varying from three weeks to six months. Orth's caused a slight increase in volume, and there was no change after one week. The Ohlmacher caused the least change and equilibrium was reached in twenty-four hours.

Sugita,† in preparation for an intensive study of the growth of the cerebral cortex and of the pyramidal cells in the albino rat, compared the effects on brain weight and histological structure of the following fixatives: (1) Bouin's fluid, (2) 10 per cent formalin, (3) 95 per cent alcohol, (4) Müller's and Orth's fluids, (5) Ohlmacher's modification of Carnoy. Specimens were imbedded in both paraffin and celloidin for comparison. He found the Bouin's fluid best adapted for his purposes, inasmuch as there was no significant change in total weight or volume, and the original shape was quite well preserved though a slight shrinkage occurred, no matter what the age of the brain. The contours of the nuclei were rounded, and the Nissl substance showed well after staining. For each brain he used 20 c.c. of Bouin's fluid, and fixed for twenty-four hours at room temperature (or two hours at 37°C.); washed in running water, twenty minutes; into 20 c.c. 80 per cent alcohol, twenty-four hours; into 90 per cent alcohol, twenty-four hours; sliced pieces 2 mm. thick, and placed in absolute alcohol, six hours; xylol, one and one-half hours at room temperature; xylol-paraffin, one and one-half hours, at 37°C.; paraffin, two hours, in oven at 56°C.; stained with carbolthionin two hours, washed slightly in running water, 70 per cent alcohol, 80 per cent alcohol, 90 per cent alcohol, absolute alcohol, xylol, balsam.

This method of preparing nervous tissues of the albino rat for growth studies has been followed by a number of investigators at The Wistar Institute of Anatomy, Donaldson and Nagasaka,‡ Ping,§ Nittono,‖ and Wang.¶

Every step in the preparation of nerve tissue after fixation still further alters its dimensions. The dehydrating fluids cause shrinkage as does also the imbedding process, especially in paraffin. The aqueous solutions through which the tissues are passed tend to increase the volume, while sectioning and affixing to the slip, especially when the temperature is

* Holt, C. M. *J. Comp. Neurol.*, 1917, xxvii, 201.
† Sugita, N. *J. Comp. Neurol.*, 1917, xxviii, 511; 1918, xxix, 119.
‡ Donaldson, H. H. and Nagasaka, G. *J. Comp. Neurol.*, 1918, xxix, 529.
§ Ping, C. *J. Comp. Neurol.*, 1921, xxxiii, 281.
‖ Nittono, K. *J. Comp. Neurol.*, 1923, xxxv, 133.
¶ Wang, C. C. *J. Comp. Neurol.*, 1927, xliii, 201.

high, cause shrinkage, as do some of the dyes used for staining, and this shrinkage is increased by the dehydration preceding mounting.

By making linear measurements at definite points in the fresh material and at the same points on the imbedded specimen, and by comparing these with the measurements made on the section after mounting, an estimate as to the changes in size can be obtained.

All size observations should be reduced to those for the fresh material, for only in this way can the results obtained by different workers, and from different animals, be properly compared.

V. Methods for Special Structures in Cell-bodies

1. Nissl Bodies. Nissl bodies are readily demonstrated in the cytosomes of nerve-cells by fixing in 95 per cent alcohol and staining the sections, after paraffin imbedding and cutting, in a 1 per cent aqueous solution of basic aniline dyes, such as methylene blue, toluidine blue, thionin, or cresyl violet.

The Nissl bodies are not seen in the living cell, but are demonstrable in the nerve cells of animals twenty-five seconds after death by decapitation (Heldt*). From its living, semi-fluid, diffuse state the Nissl substance changes into the form of granules, which have more or less characteristic shapes and patterns of arrangement in different types of nerve cells. The size and structure of the Nissl granules are largely dependent upon the treatment of the cells in preparation, and especially upon fixation. Nerve cells, by some fixations, show only a diffuse mass of basophilic substance in their cytosomes, with no distinct units. Similar nerve cells treated by other methods show definitely formed aggregations of granules, distributed throughout their cytosomes. Hopkins† has studied the appearance of Nissl substance in the motor cells of the ventral gray columns in the spinal cord of the adult albino rat, following variations in fixation. He finds that by the addition of 2, 5, or 10 per cent of acetic acid to 100 parts of 95 per cent alcohol, the boundaries of the Nissl bodies are more sharply defined than in 95 per cent alcohol alone. Similar results are given by fixation in equal parts of 10 per cent formalin and 95 per cent alcohol, and also by fixation in 10 per cent formalin to which has been added 2 or 5 per cent of acetic acid. In these tests the fixation period was forty-eight hours. As varying pictures are given by different methods, it follows that in making comparisons one method should be selected and employed throughout. In studying Nissl granules in ten ganglia of cranial and spinal nerves of dogs, S. L. Clark‡ fixed overnight in a solution of 5 per cent acetic acid in 95 per cent alcohol, and imbedded in paraffin. Sections were cut 7 μ to 10 μ,

* Heldt, T. J. *J. Comp. Neurol.*, 1913, xxiii, 315.

† Hopkins, A. E. *Anat. Record*, 1924, xxviii, 157.

‡ Clark, S. L. *J. Comp. Neurol.*, 1926, xli, 423.

and stained with a 1 per cent aqueous solution of toluidine blue. In this material seven types of Nissl-granule arrangement were identified.

The Nissl granules can be demonstrated for some time after death. Malone* used an entire human medulla, removed from the cranium seventeen hours post mortem. This was fixed in 95 per cent alcohol for several days, imbedded and sectioned in paraffin.

Sample method for Nissl bodies.

1. Fix tissue, just removed from body, in 95 per cent alcohol to which has been added 5 per cent acetic acid, for one to two days.
2. Imbed in paraffin or parlodion. If latter is used it should be removed from sections before staining.
3. Stain for six hours to overnight in a 1 per cent aqueous solution of one of the following: thionin, toluidine blue, cresyl violet, methylene blue.
4. Rinse in water.
5. Dehydrate for one minute each in 80 per cent and 95 per cent alcohol.
6. Pass quickly through absolute alcohol, which removes the stain rapidly and differentiates the granules.
7. Clear in xylol.
8. Mount in damar or clear balsam.

Neutral red in dilute solution, well ripened, was found by Johnston† to be especially useful after formalin fixation. The neutral red must be ripened, in 1 per cent aqueous solution, for one, two or four years. For use it is diluted to $\frac{1}{4}$ or $\frac{1}{10}$ of 1 per cent, and this diluted stain may be used repeatedly. It is advised to clear the sections in 1 part xylol with 2 to 3 parts of castor oil. Good results were obtained in sections 50μ thick, through the whole brain of the newborn child. It may also be used after the Cajal or Bielschowsky methods, on alcohol- or formalin-fixed material. Morgan‡ has reported a method of rapidly ripening the neutral red, by means of the colon bacillus.

When the processes of nerve cells are injured there are chemical and morphological changes in the Nissl substance. When the cell does not degenerate completely as the result of the injury, the Nissl substance gradually reforms into the normal Nissl bodies. In general, the same methods are used for showing degenerative and regenerative changes as for the normal Nissl bodies. Nicholson§ has found in the albino rat, after ligation of the axons of the hypoglossal nerve, that degeneration progressed from the first to the fifteenth day, and that regenerative processes took place and steadily progressed from the sixteenth to the forty-fourth day. Papez,‖ studying the subdivisions of the facial nucleus by the method of chromatolysis, removed the tissues to be examined from rat, guinea pig, cat

* Malone, E. F. *J. Comp. Neurol.*, 1923, xxxv, 205.
† Johnston, J. B. *Anat. Record*, 1916, xi, 297.
‡ Morgan, L. O. *Anat. Record*, 1926, xxxii, 283.
§ Nicholson, F. M. *J. Comp. Neurol*, 1923, xxxvi, 37.
‖ Papez, J. W. *J. Comp. Neurol.*, 1927, xliii, 159.

and dog, at the thirteenth to the seventeenth day after operation. Windle,* after removal of the pulp from the teeth of dogs, found chromatolytic changes in certain cells of the Gasserian ganglion after twelve to fourteen days. Both Papez and Windle fixed in Carnoy's (van Gehuchten's) fluid; the former stained with methylene blue, the latter with toluidine blue.

2. Neurofibrils. *a. Cajal's Reduced Silver Methods.* The central idea of Cajal's methods is the application of photographic developers to tissues which have been treated with silver nitrate. In all the methods except the original there is a preliminary fixation before the immersion in silver nitrate. The neurofibrils are seen within the neurons, and the axons, dendrites, and telodendria are impregnated with a reduced silver compound. The outside of the blocks is usually over-impregnated, and in only a limited zone are the elements seen at their best. Many modifications have been introduced into the original method to adapt it for special purposes. These consist principally in the chemicals used in the preliminary fixation, e. g., absolute alcohol, ammoniated alcohol, formalin followed by ammoniated alcohol, pyridine, chloral hydrate, pyridine and chloral hydrate together, etc. The first six methods given here were published by Cajal† in 1910 (the original method appeared in 1903). These have since been utilized for many studies, which have appeared in Cajal's journal. Other methods for use on tissues requiring decalcification and for frozen sections have also been devised. The following are the procedures in current use at the Instituto Cajal, from personal observation in 1928. The chemicals should be chemically pure, especially in the fluids used for the preliminary fixations. The alcohol for fixation, both when used alone and in the ammoniacal alcohol fixation, may be absolute or 96 per cent, but should be high-grade, as for analysis. The temperature of the oven in which the tissues remain in the silver nitrate solution should be at or slightly below body temperature, 35°–39°C.

Experience has shown that the selection of the formula to be tried depends on the part of the nervous system being studied, on the age of the animal, and to a certain extent on the species.

Spinal Cord. All the formulas are applicable to the spinal cord. The neurofibrils of the motor neurons of the ventral horns are shown well by Method III, especially in young animals, during the first two weeks after birth. For myelinated fibers, large and small, Methods II and VI are effective; for the buttons or *Endfüsse* of Held-Auerbach, the myelinated plexus of the gray matter and the myelinated fibers of the white matter, Methods III, IV, and V.

Cerebellum. The cerebellum responds to the different formulas with a considerable variety of reactions. For the adult Purkinje cells, and the terminal pericellular arborizations of the basket cells, Method III; for

* Windle, W. F. *J. Comp. Neurol.*, 1927, xliii, 347.

† Cajal, S. Ramón y. *Trab. Lab. Investig. Biolog. de la Univ. Madrid.* 1910, viii, 1.

adult mossy fibers, Methods IV and VI; for climbing fibers, parallel fibers, axons of stellate cells, Method VI (this method is especially good for these structures in small adult animals). The new method of Cajal for use on frozen sections (alcohol-silver-pyridine)is excellent for the baskets around the Purkinje cells. For embryos, especially the younger stages, Method V. For the granule cells, Methods I and II, and sometimes III.

Cerebrum. In the cerebrum Method I is advantageous for the pyramidal cells, especially the medium and small cells, particularly in young animals, as cat and dog, eight to thirty days; for unmyelinated and myelinated fibers, of medium and larger size, Methods II and III, (diminishing the quantity of ammonia in III); for fine nerve plexuses, Methods IV, V, and VI.

Neuroblasts. For the demonstration of neuroblasts and their processes, avoid formulas with a base of formalin. The best results are given with alcohol, and with pyridine. These are useful with all the vertebrates, especially with embryos of birds and of fish. For more advanced stages of embryos and fetuses of mammals, however, Methods III and VI and alcohol with accelerator may be used (rabbits from the tenth to eleventh day until birth, and chicks from the fifth day of incubation).

Ganglia. For sympathetic ganglia are advised Methods III, IV and V. The visceral ganglia (plexuses of Auerbach and Meissner, etc.) are more difficult to color. For these Castro has obtained good results with formulas containing chloral hydrate and urethane.

For sensory ganglia Methods II, III and V are used.

Method I. 1. Without preliminary fixation, place pieces of tissues, not over 3mm. in maximum thickness, in 1½ per cent aqueous solution of silver nitrate, for three to five days, at 37°c. in the dark. The silver solution should be abundant, 80 to 100 c.c. for 2 or 3 pieces of tissue, and should be kept in glass bottles, with ground stoppers or with corks paraffined to prevent evaporation. The impregnation is sufficient when the pieces have assumed the color of tobacco.

2. Rinse the tissues in distilled water.

3. Transfer to the following reducing fluid, for twenty-four hours.

Pyrogallic acid or hydroquinone	1 to 2 gm.
Formalin, full strength	5 to 10 c.c.
Distilled water	100 c.c.

4. Rinse in distilled water.

5. Harden in absolute alcohol, for twenty-four hours.

6. Imbed in parlodion or paraffin, section, clear, and mount in balsam or damar. Parlodion allows thicker sections (20 μ) and there is less shrinkage.

Before mounting the sections, it often improves the clearness of the preparations to tone them in a bath of gold chloride, as follows:

Water.
Yellow chloride of gold 1 to 500 parts of water for fifteen to thirty minutes.
Water.
Hyposulphite of soda 5 or 10 per cent for one-half minute.
Water.
Dehydrate etc. and mount in balsam.

In general, this method has the defect of having the impregnation less penetrating than the following methods, in which a preliminary fixing agent is employed. It is applicable to the brains of small animals or to those of fetuses and the very young of large animals. It gives good results with the medulla, pons, cerebellum, cerebrum of fetal and neonatal animals, and with the cerebellum and cerebrum of adults. In invertebrates, especially with Hirudo, good results have been obtained using 6 per cent silver nitrate.

Method II. 1. Fix in alcohol, absolute or 96 per cent for twenty-four hours. The alcohol should be pure, containing no acetone, glycerin, or other extraneous substances. In place of ethyl alcohol one may use methyl, propyl, butyl, or allyl alcohol.

2. Cut out pieces not over 2½ mm. thick and place them in the 1½ per cent aqueous solution of silver nitrate, for five to seven days, in the dark at 28 to 35°C.

3. Rinse in distilled water.

4. Reduce, etc., as in the preceding method.

This colors splendidly both myelinated and unmyelinated nerve-fibers, pericellular arborizations and large and medium-sized nerve cell-bodies. Best results are with cerebellum and cerebrum. Impregnates very well also the motor and sensory nerve-terminations (corpuscles of Pacini, Meissner, Krause, Merkel, etc.). Gives good results also with nerves in course of regeneration, with young embryos (embryos of chicks from sixty hours onward) and with young fish.

A variant of this method is to use as fixing agent a mixture of alcohol with an hypnotic (veronal, chloral hydrate, etc.) e. g.:

Alcohol 96 per cent or absolute	50 c.c.
Chloral hydrate or veronal	1 gm.

This requires only five days in the silver nitrate and is said to increase the constancy and regularity of the reaction. With very young embryos, however, the addition of the accelerators, e. g., veronal, does not give as good results as does the alcohol alone.

Material which has been for a long time in alcohol may be rejuvenated by chloral hydrate or veronal. Thus, pieces of human cerebrum and cerebellum which have been in 95 per cent alcohol for a whole year and which then give but a weak coloration, acquire a greater electivity after treatment with chloral hydrate or veronal. The same effect may also be obtained with ammoniated alcohol.

Method III. 1. Fix for twenty-four hours in

Absolute or 96 per cent alcohol	50 c.c.
Ammonia (conc. 22)	1 to 12 drops

It is important to modify the quantity of ammonia according to the part of the nervous system to be studied. For spinal cord and medulla, 8 to 12 drops; for cerebrum, 1 to 3 drops; for cerebellum, 4 drops; for peripheral nerve-endings, 2 to 3 drops. If the quantity of ammonia is excessive, the coloration is pale. In order to insure uniform size of

the drops one should use a very fine tube, as a 1 c.c. pipette, calibrated to hundredths of a cubic centimeter.

2. Mop off with filter paper.
3. Place in silver nitrate, etc., as before.

This method is particularly good for the neurofibrils of spinal cord and the ganglia of neonatal dogs, cats and rabbits. Good results also with the adult cerebrum and cerebellum and sympathetic system. For the last, especially in man and other large mammals, this formula is the best, according to Castro.*

In the fixation, excessive contraction may be avoided by first placing the pieces in 70 per cent alcohol for six hours, then in 85 per cent alcohol for an hour or two, and finally in the ammoniated alcohol.

Method IV. 1. Fix for six to twelve hours in 15 per cent formalin.
2. Wash in running water, six hours or more, to remove formalin completely.
3. Continue fixation in ammoniated alcohol, for twenty-four hours:

Alcohol, 96 per cent or absolute	50 c.c.
Ammonia (conc. 22)	5 drops

4. Mop with filter paper.
5. Impregnate four to five days in the silver nitrate at 38 to 40°c.

Good results with the unmyelinated fibers of the central nervous system and the pericellular arborizations, especially of adult animals. It colors well the mossy fibers of the cerebellum. For the fixation one may also use a mixture of formalin and alcohol.

Method V. 1. Fix for twenty-four hours in pyridine undiluted, or
in pyridine, diluted with an equal quantity of distilled water, or
in 40 parts of pyridine with 20 parts of 95 per cent alcohol.
It is best to use the diluted pyridine with adult tissues, to avoid undue distortion and vacuolization.

2. Wash in running water, twelve to twenty-four hours, until odor is gone.
3. Transfer to 95 per cent or absolute alcohol, six to twelve hours.
4. Impregnate in the usual way, four to five days.
5. Reduce, etc., as in previous methods.

This gives good results especially in young embryos (chick embryos from end of fifth day, rat from 8 mm., rabbit from 15 mm.) and in nerves in course of regeneration; in adults it colors by preference the fine myelinated fibers; it colors strongly the neurofibrils. Cajal now usually employs the pyridine at 70 per cent, and obtains good results in embryos on account of the great contrast and power of penetration. Moreover, it is excellent for nerve terminations and for the phenomena of regeneration. It, however, has the disadvantage of being somewhat inconstant, and of not revealing in the case of regeneration the earliest formations. But when properly treated the regenerating fibers are colored intensely. The intensity of color

* de Castro. *Trab. Lab. Investig. Biolog. de la Univ. de Madrid*, 1921, xix, 241–340.

after treatment with the silver nitrate is in general paler than that with the other formulas.

Method VI. 1. Fix for twenty-four hours in:

Chloral hydrate	5 to 6 gm.
Absolute alcohol	25 c.c.
Distilled water	75 c.c.

(The original Cajal formula was chloral hydrate 5 gm., water 50 c.c., but Castro has suggested the addition of the alcohol, to restrain the swelling of the tissue.)

2. Wash rapidly in distilled water.

3. Treat with ammoniated alcohol, twenty-four hours (absolute alcohol 50 c.c. ammonia 4 drops).

4. Impregnate, etc., as before.

This gives the best coloration for motor end-plates, pericellular arborizations, cerebellum, etc. The addition of the ammonia to the alcohol is necessary in order to render alkaline the acid fluid of the chloral hydrate, because the reaction takes place always in a slightly alkaline medium.

In certain cases it is advantageous to increase the quantity of alcohol in the fixative, as for example:

Chloral hydrate	5 gm.
Absolute alcohol	40 c.c.
Distilled water	40 c.c.

By this was obtained good impregnations of terminations in muscles in crayfish. Another formula which gave good results with crayfish is:

Chloral hydrate	5 gm.
Pyridine	20 c.c.
Absolute alcohol	40 c.c.
Distilled water	40 c.c.

In addition, there is the formula of Castro with urethane:

Urethane	2 gm.
Absolute alcohol	40 c.c.
Distilled water	40 to 50 c.c.

Both urethane and chloral hydrate color at one time the nerve-fibers and the nuclei.

(1) Modifications of Cajal's Methods for Calcified Tissues.

Cajal and Castro* have devised several formulas for combining decalcification with fixation:

(a) *Cajal.*

1. Fix and decalcify, usually one to two days, in

Formalin	14 c.c.
Distilled water	86 c.c.
Nitric acid	3 c.c.

2. Wash carefully for one day.

3. Treat with ammoniated alcohol, or a mixture of alcohol with pyridine (see Method v) for one day.

* de Castro, F. *Trav. Lab. Recher. Biolog. de la Univ. de Madrid,* 1925, xxiii, 427–446.

4. If alcohol and pyridine are used be careful to wash well before passing into the silver nitrate. If ammoniated alcohol is used mop off.

5. Impregnate, etc. as before.

(b) *Castro.*

1. Fix and decalcify in the following (until lime salts dissolve, one to two days):

Chloral hydrate	5 to 6 gm.
Absolute alcohol	25 to 40 c.c.
Distilled water	40 to 75 c.c.
Nitric acid	3 c.c.

2. Wash carefully, for twenty to twenty-four hours.

3. Treat with ammoniated alcohol (4 to 5 drops of ammonia in 50 c.c. absolute alcohol) one day.

4. Proceed as before.

(c) *Castro.*

1. Fix and decalcify in

Urethane	2 gm.
Absolute alcohol	40 to 60 c.c.
Distilled water	40 c.c.
Nitric acid	3 c.c.

2. Wash carefully and proceed as before.

(2) Modification of Cajal's Method, for Fish Brains.

Bartelmez,* using Salmo and Ameiurus, fixed entire larval heads or brains of adults in acetic alcohol. For larval forms, a mixture of 10 parts of absolute alcohol with 1 part of glacial acetic acid gave the best results. For adults he fixed in

Absolute alcohol	19 parts		Absolute alcohol	19 parts
Glacial acetic acid	1 part	or	Glacial acetic acid	2 parts
			Chloroform	10 parts

Fixation for twenty minutes is usually sufficient; it should not be more than one and one-half hours; otherwise the material becomes too brittle. Rinse in 80 per cent alcohol, followed by distilled water, and then place in the silver nitrate solution, in the dark, at 35 to 40°c. The solution is changed each day, and the strength altered thus: first day 1 per cent silver nitrate; second day 1.5 per cent; third day 2 per cent; fourth day, 1 per cent again, and repeating as before, never going above 2 per cent silver nitrate. The total time in the silver baths varies from 3 to 8 days, usually best at four to five days, when the material becomes brown. Reduction is by pyrogallol-formalin as in Cajal Method 1, with final imbedding in paraffin. This gives a preparation showing some cells in every nucleus stained with all their processes including the axon; and in many the endings of the eighth nerve in the internal ear were well shown.

(3) Modifications of Cajal's Methods for Large Pieces. Gurdjian† has found the Cajal methods applicable to relatively large pieces. By the

* Bartelmez, G. W. *J. Comp. Neurol.*, 1915, xxv, 87.

† Gurdjian, E. S. *J. Comp. Neurol.*, 1927, xliii, 1.

following modifications it was possible to impregnate successfully a whole brain stem of a seventeen year old boy, brain stem of a large adult dog, adult rat brains, pigeon brains, etc. To insure more rapid and uniform fixation the blood vessels of the part may be injected with the fixing fluid, but this is not necessary with brains of albino rats or others of similar size.

1. Fix in mixture of 100 parts of 95 per cent alcohol, with 1 part of concentrated ammonia for ten to twenty days (change daily). Mop off before putting in the next solution.
2. Transfer to a 2 to 5 per cent solution of chloral hydrate in 95 per cent alcohol, 3 to 5 days (change daily). Rinse in distilled water.
3. Place in ammoniated alcohol again, two to three days (two to three changes).
4. Impregnate in 0.75 per cent aqueous solution of silver nitrate, for two to four weeks, in the dark, at about 30°C. (change twice a week).
5. Reduce in

Pyrogallic acid	5 parts
Formalin	5 parts
Distilled water	100 parts

Leave here for a week to ten days, and longer if necessary.

(4) Modifications of Cajal's Methods for Frozen Sections. The Cajal methods, used on blocks of tissue, have the advantage of giving a regular impregnation and a great variety of reactions; they have the disadvantage of demanding a relatively long time, and, in the case of embryological research, of being limited to too small pieces.

By the use of a protective colloid in the reducing agent, Liesegang has been able to adapt the method for frozen sections.

Liesegang's modification for frozen sections.
1. Fix in 12 per cent formalin.
2. Make frozen sections.
3. Transfer to silver nitrate 0.75 per cent, for some hours, until they become brown.
4. Reduce in

Hydroquinone, 0.5 per cent aqueous solution	1 part
Silver nitrate, 0.75 per cent aqueous solution	1 part
Gum arabic, 50 per cent aqueous solution	1 part

In a few seconds the sections become dark, and the neurofibrils are colored.

(5) *Cajal's* modification for frozen sections.

Cajal* has developed still another method for use with frozen sections. It is rapid in execution, and is particularly applicable to the cerebellum.

Formula with alcohol, silver and pyridine.
1. The blocks of large brains or entire small brains are fixed in 20 per cent formalin, for three days or more. Pieces which have been in the fixature more than a year still give good results.
2. Frozen sections are cut 30–40 μ.
3. Wash for several minutes in distilled water.

* Cajal, S. Ramón y. *Trav. Lab. Recher. Biol. Univ. de Madrid*, 1926, xxiv, 217.

4. Place sections for four to six hours in the following mixture,

Silver nitrate, 2 per cent	12 c.c.
Pyridine, pure (e.g. Kahlbaum)	7–10 drops
Alcohol 97 per cent	5–6 c.c.

The sections become light brown in color. This reaction takes place at the room temperature, but is more rapid and certain in the oven.

5. Rapid passage, two to four seconds' duration, through absolute alcohol, 8 to 10 c.c., in a glass or porcelain dish. In order to avoid excessive extraction of the nitrate, only 2 or 3 sections should be passed through at one time.

6. Reduction, one to three minutes in the following mixture, which may be kept in a stock bottle.

Hydroquinone	0.30 gm.
Distilled water	70 c.c.
Formalin	20 c.c.
Acetone	15 c.c.

7. Wash in a large quantity of water.

8. It is best to tone the sections in gold chloride, yellow chloride of gold, 1 to 500 parts of water; water; hyposulphite of soda, 5 or 10 per cent, one-half minute.

9. Wash; mount on slips; dry with filter paper; alcohol 95 per cent and absolute; xylol; balsam or damar.

All manipulations should be done with glass hooks.

By this method are colored intensely the arborizations of the basket cells around the Purkinje cells, and the mossy and climbing fibers of the cerebellar cortex, the calyces of Held in the nucleus of the trapezoidal body and the terminations in the ventral ganglion of the acoustic nerve.

b. Bielschowsky's Silver Methods for Neurofibrils. In the Bielschowsky methods formalin-fixed tissues are treated first with silver nitrate; next, a freshly prepared ammoniacal silver salt, and then with a reducing agent—formalin. As a result of the reduction there follows a silver impregnation of the neurofibrils, axons and dendrites. Other structures, as fibers of connective tissue and neuroglia, may also be impregnated. Here, as in all methods involving the reduction of gold and silver salts, the glassware must be physically and chemically clean, and the sections or blocks should be manipulated with glass rods, or with forceps having their points paraffin-coated.

The methods are applicable to thin pieces of tissue which have been recently fixed in formalin, and also to similar pieces taken from brains or spinal cords which have already been preserved for a long time (five to ten years), in formalin; and they succeed both on human tissues from autopsy and on animal tissues. They are therefore of great value to the neuropathologist. The impregnation may be carried out either with frozen sections, or with pieces which are later imbedded and sectioned in paraffin or celloidin. In the method with pieces, there is a period of immersion in pyridine before the silver baths.

The methods of Bielschowsky are also valuable for motor and sensory nerve-endings, including those in the organs of special sense.

(1) Bielschowsky's Silver Method on Frozen Sections.

1. Fix thin pieces in neutral formalin 10 to 15 per cent. If the slices are not over 3 mm. in thickness, fix for one day or more; for larger pieces the time must be prolonged according to their mass until fixation is complete; one may also use thin pieces cut from brains already fixed in formalin.

2. Wash an hour or more in running water, to remove the formalin.

3. Prepare frozen sections, as thin as possible compatible with handling.

4. Wash in distilled water (1 to 2 hours) with several changes.

5. Transfer from the distilled water (using glass instruments) to silver nitrate, 2 to 3 per cent where they remain twenty-four to forty-eight hours in the dark.

6. Rinse quickly in distilled water, and place in a fresh ammoniacal silver solution, prepared as follows:

To 5 c.c. of 10 per cent aqueous solution of silver nitrate add 5 drops of 40 per cent aqueous sodium hydroxide; a dark brown precipitate of silver oxide is formed. To this is added, drop by drop, concentrated ammonia; the solution is shaken vigorously after the addition of each drop; continue adding the ammonia until the precipitate has practically disappeared, but there must be no excess of ammonia. The solution contains ammoniated silver oxide and nitrate. Then add distilled water, until there is altogether 20 c.c.

Leave sections in this solution ten to twenty minutes (or less) until they are deep brown.

7. Pass quickly through distilled water and place in the reducing fluid (20 per cent formalin made up with tap water), for five to thirty minutes, until no more whitish clouds are given off. The sections quickly change to a slatey-gray or blackish color.

8. Wash in distilled water five to ten minutes.

9. Transfer to the following gold solution:

1 per cent gold chloride, aqueous	2 to 5 drops
Distilled water	10 c.c.
Glacial acetic acid	2 to 3 drops

The general tone becomes lighter, while the impregnated structures become deep black.

10. Wash quickly in distilled water.

11. Fix in 5 per cent solution of sodium thiosulphate for thirty to sixty seconds.

12. Wash thoroughly in running water for several hours, dehydrate, clear in carbol-xylol (1 to 10), and mount in balsam.

(2) Bielschowsky's Silver Method with Pyridine for Pieces.

1. Fix in neutral formalin 10 per cent, as in the previous method.

2. Wash an hour or more, finally rinsing in distilled water.

3. Transfer to pyridine, pure, for three to four days.

4. Wash thoroughly in many changes of distilled water to remove excess of pyridine.

5. Place in 3 per cent aqueous solution of silver nitrate at 37°c., for three to five days, in the dark.

6. Rinse in distilled water, and place in a fresh ammoniacal silver solution (⅕ the strength of that in the previous method) prepared as follows:

To 10 c.c. of 10 per cent aqueous solution of silver nitrate add 5 drops of 40 per cent aqueous sodium hydroxide. The precipitate thus formed is nearly all dissolved by the addition of concentrated ammonia drop by drop, as before, the solution being well shaken after each drop is added. Then add distilled water until there is altogether 50 c.c.

Leave here for twenty-four hours.

7. Wash for two hours in several changes of distilled water and place in the reducing fluid (20 per cent formalin) for several hours.

8. Rinse in distilled water, dehydrate in graded alcohols, imbed in paraffin, section.
9. Tone the sections, on the slides, with the gold chloride solution, as before.
10. Wash briefly in distilled water and fix in the thiosulphate solution.
11. Wash thoroughly, dehydrate, clear and mount in balsam.

This gives excellent results, is somewhat simpler than the previous method, and has the advantage of affording sections in serial order. The impregnated neurofibrils, dendrites, axons, etc., are black on a yellowish brown background. The methods of Bielschowsky are also valuable for both motor and sensory nerve endings, including those in the organs of special sense.

3. Internal Reticular Apparatus of Golgi (Golgi Net). The material should be fresh, and not used more than two hours after death. Fetal and young mammalian tissues are preferable. The original Golgi method has been largely replaced by the following and other modifications.

Cajal's uranium-formalin method, for Golgi net.

1. Fix pieces 2 to 2½mm. thick for eight to twenty-four hours in the following:

Uranium nitrate	1 to 2 gm.
Neutral formalin, full strength	15 c.c.
Distilled water	85 c.c.

The duration of fixation should vary with the material; often in young animals a few days old it is necessary to leave for twenty-four to thirty-six hours.

2. Rinse in distilled water less than a minute.
3. Transfer to 1½ per cent silver nitrate (if the pieces are very small, 0.75 per cent) for thirty-six to forty-eight hours.
4. Rinse for some seconds in distilled water.
5. Reduce in the following reducing fluid eight to twelve hours.

Hydroquinone	1 to 2 gm.
Formalin, full strength	15 c.c.
Distilled water	85 c.c.
Anhydrous sulphite of sodium	0.15 gm.

6. Wash briefly in running water.
7. Dehydrate in graded alcohols, leaving the pieces not more than one to two hours in the 90 per cent and absolute alcohols.
8. Imbed in parlodion or paraffin.

In successful preparations the Golgi net shows as a black network on a bright yellow background.

Da Fano's modification is to use cobalt nitrate instead of uranium nitrate as the fixing agent, making it as follows:

Cobalt nitrate	1 gm.
Formalin	15 c.c.
Distilled water	100 c.c.

The best coloration of the Golgi apparatus is obtained in the cat, dog and rabbit, in animals fifteen to twenty days old. By augmenting the quantity of the formalin (up to 20 per cent) in the fixing fluid, as proposed

by Penfield,* or by excluding the sodium sulphite in the reducer, splendid coloration has also been obtained with adult tissues. The zone of useful reaction is always small (0.2 to 0.5 mm.), for it is not possible to use pieces thicker than 3 mm. The sulphite is not a necessity, but it appears that a little alkalinity favors the coloration of the Golgi net. One may counterstain with hematoxylin (Ehrlich's or Bohmer's) or a basic aniline dye, as gentian violet, thionin, methylene blue, safranin, etc. The formalin should not be acid; to prevent this use calcium carbonate or pulverized gypsum. When the coloration is intense but somewhat granular one may try adding to the fixative ethyl or methyl alcohol, thus:

Uranium nitrate	1 gm.
Alcohol, ethyl or methyl	30 c.c.
Neutral formalin	15 to 20 c.c.
Distilled water	80 c.c.

With this formula successful preparations show the Golgi net dark gray on a bright yellow background.

4. Mitochondria. The methods used with the other tissues are applicable also to the nervous system (p. 198). The Champy-Kull method gives good results with mammalian nervous tissues (p. 201).

5. Glycogen. The glycogen in the nerve-cells may be fixed by alcohol at any concentration from 67 per cent to absolute, but it is soluble in alcohol less than 67 per cent. Plenty of alcohol should be used, about 50 times the bulk of the tissue. Material may be decalcified, after fixation in alcohol, with 3 per cent nitric acid in 67 per cent alcohol. Another fixing fluid is picric-alcohol, made of 500 c.c. of 67 per cent alcohol with 1 gm. of picric acid. Leave in this fluid for twelve to twenty-four hours, then in 67 per cent alcohol twelve to twenty-four hours, and then in 82 per cent alcohol for a day or more. Imbed in paraffin or in combined paraffin and parlodion, and cut sections at 10 μ to 15 μ.

Stain with iodine. A useful formula is:

95 per cent alcohol	150 c.c.
Distilled water	150 c.c.
Iodine, crystals	1.5 gm.
Potassium iodide	3 gm.
Sodium chloride	1.5 gm.

For staining spread the paraffin sections with the staining fluid instead of water, and leave for two to three minutes. If the paraffin is not melted off the sections may be restained at any time. Such preparations are useful only for low-power observation. They may be mounted in vaseline as follows: Stain without removing the paraffin, then dry for half an hour on top of the oven or in the air. When thoroughly dry, dissolve off the paraffin in xylol and cover with yellow vaseline. It is best to seal around the

* Penfield, W. G. *Anat. Record*, 1921, xxii, 57.

margin of the cover glass with shellac. The stain lasts two to ten years. The sections may also be mounted with heated balsam, without covers. This is better for study with the highest powers of the microscope, but the stain does not last as long as with the vaseline (Gage).*

6. Trophospongium of Holmgren. Several methods may be used.

Ross,† studying nerve-cells of the abdominal ganglia of the crayfish (*Cambarus*) used Bensley's acetic-osmic-bichromate acid fuchsin method, or fixed in Zenker's fluid and stained with Mallory's connective tissue stain.

Penfield,‡ showing that the trophospongium and the internal reticular apparatus of Golgi were separate structures, stained the former with iron hematoxylin.

7. Centrosomes. For the first demonstration of centrosomes in the nervous system, Lenhossek§ used several methods: Flemming's fixing solution, followed by thionin; Hermann's fluid, with magenta red; sublimate with Heidenhain's iron hematoxylin and Bordeaux red. These methods are still in use, especially the staining with Heidenhain's iron hematoxylin, following Flemming's fluid or other fixatives.

Hatai‖ demonstrated their presence in certain nerve-cells of the albino rat. In addition to other methods, he devised one of his own which gave him good results. Its formula is as follows:

Mercuric chloride, saturated solution in formalin	30 c.c.
Glacial acetic acid	50 c.c.
Physiological salt solution	15 c.c.

After six to twelve hours' fixation, the thin pieces were washed in running water for four to five hours, transferred to 30 per cent alcohol, and then by graded alcohols were dehydrated and imbedded in paraffin. For staining he used toluidine blue or thionin in saturated aqueous solution; cleared in xylol, mounted in balsam. By this method centrosomes were shown in the large pyramidal cells of the cerebral cortex, Purkinje cells and spinal ganglion cells of the adult albino rat, and also in the ventral horn cells of the spinal cord and in the cells of the nucleus dentatus of the newborn rat.

Rio-Hortega¶ has successfully applied to the study of centrosomes the neuroglia stain with tannin-ammoniacal silver, which he terms his "first variant of the method of Achúcarro."

8. Pigment. The endogenous pigment in nerve-cells is usually of a bright golden color, in the form of granules, generally massed in one region of the cell-body, and is readily recognized by its natural coloration.

* Gage, S. H. *J. Comp. Neurol.*, 1917, xxvii, 451.

† Ross, L. S. *J. Comp. Neurol.*, 1915, xxv, 523.

‡ Penfield, W. G. *Brain*, 1921, xliii, 290.

§ Lenhossék, M. v. *Arch. f. mikr. Anat.*, 1895, xlvi, 345.

‖ Hatai, S. *J. Comp. Neurol.*, 1901, xi, 25.

¶ Del Rio-Hortega, P. *Trab. Lab. Investig. Biolog. Univ. de Madrid*, 1916, xiv, 117–153.

VI. Methods for Normal Myelin

1. Weigert's Myelin-sheath Methods. These methods are used principally on formalin fixed tissues of the central nervous system to demonstrate the fiber-tracts and to show the arrangement of the gray and white matter. Proper fixation and mordanting are necessary for the success of the staining. The nervous tissue, either in the form of the entire brain or cord or small portions of them, or of short pieces of peripheral nerves, is fixed in 10 per cent formalin. The duration of fixation for small pieces is a week or more; for the entire human brain one to two months or longer. If the tissues are to be set aside for further preparation at a later time it is best to keep them in neutral 10 per cent formalin.

The following modification has been found useful on both human and animal tissues. This requires the employment of four special solutions: the primary mordant (A), the secondary mordant (B), the hematoxylin stain (C) and the differentiating fluid (D) made respectively as follows:

A. Primary mordant (Weigert's rapid mordant)

Potassium dichromate	5 gm.
Fluorchrome	2 gm.
Distilled water (boiling)	100 c.c.

B. Secondary mordant (copper mordant, Weigert's neuroglia mordant)

Copper acetate	5 gm.
Acetic acid, 30 per cent or 36 per cent	5 c.c.
Fluorchrome	2.5 gm.
Distilled water	100 c.c.

In making this solution, boil the fluorchrome and water in a covered vessel, turn off the gas, add the acetic acid, then the copper acetate, stir briskly until the latter is dissolved and allow to cool. The solution remains clear.

C. Hematoxylin stain.

10 per cent solution of hematoxylin in abs. alc. (stock sol.)	10 c.c.
Lithium carbonate (saturated aqueous sol.)	1 c.c.
Water	90 c.c.

It is very important that the stock solution of hematoxylin should be well ripened (two months or more, six months preferably), but the staining fluid should be made up just before using.

D. Differentiating fluid

Potassium ferricyanide	2.5 gm.
Sodium biborate (borax)	2.0 gm.
Distilled water	100.0 c.c.

After fixation the blocks of tissue are washed in running tap water overnight, before being placed in the primary mordant. Here they remain one week or longer, depending upon their size. After washing they are passed into the secondary mordant for one to several days. The blocks are then washed in water, dehydrated in graded alcohols and imbedded in parlodion. Sections are usually cut at 25μ to 50μ. They are then placed in

the staining solution overnight (twenty to twenty-four hours) where they become a dense blue-black color. After staining the sections are washed thoroughly in several changes of tap water, and left in the water overnight. They are then placed in the differentiating fluid and the decolorizing process watched. The color gradually leaves the gray matter, but is retained in the myelin sheaths of the white matter, so that the distinction between the gray and the white matter becomes more and more distinct. The time for this process is usually between twenty minutes and an hour. The differentiation is stopped by transferring the sections to water.

After differentiation the sections are to be thoroughly washed in tap water (several changes and leave overnight), dehydrated in alcohols, cleared in carbol-xylol followed by xylol, and mounted in balsam.

A variation of this method is to use the secondary mordant on sections instead of on the block. The procedure would then be: fixation, primary mordant, imbedding and cutting, secondary mordant (overnight), staining and differentiating.

Instead of lithium-hematoxylin, the following stain, Weigert's iron hematoxylin, may be used.

Distilled water	90 c.c.
10 per cent solution of hematoxylin in abs. alc. (ripened)	10 c.c.
Solution of ferric chloride, U.S.P.	4 c.c.

The myelin sheaths are stained black, whereas with the lithium hematoxylin they are stained blue.

2. Weigert Method in Edinger's Laboratory. Paula Meyer* in Edinger's laboratory, where the Weigert tradition still held, made a careful comparison, of eight variants of the method, on human brain tissue. She used for the test object, as did Weigert originally, the cerebral cortex, and especially the appearance of the supraradial feltwork. The following was selected as the most satisfactory:

After the brain has been in the 10 per cent formalin for two weeks or longer, thin slices are cut out, washed and placed in 5 per cent potassium dichromate or Müller's fluid in the oven, at not over 37°C., for a period of two weeks or more. The solution should be changed several times, when it becomes turbid, and the tissues left in it until the white matter everywhere becomes brown, not merely yellow. The blocks are dipped in water and are then placed in 70 per cent alcohol, which should be changed repeatedly, as long as the color comes out of the tissues. They are kept in a dark cupboard during this process. They are then imbedded in celloidin and sectioned. The sections are mordanted in the copper mordant (B, p. 339) for twenty-four hours, at 37°C. Or, if the blocks of tissue are small, the blocks may be mordanted before being sectioned. After being washed in 70 per cent alcohol, the sections are stained in Weigert's iron hematoxylin (see above) for overnight or twenty-four hours. They are then washed in water and differentiated with the borax-ferricyanide mixture (D, p. 339). This may be used at half strength at first, and the differentiation finished with the undiluted mixture. The sections should then remain for at least twenty-four hours in water, to which a few drops of

* Meyer, P. *Neurol. Centralbl.*, 1909, No. 7.

lithium carbonate have been added, and this solution should be changed repeatedly. The sections are then passed through 70 per cent, 95 per cent alcohol, carbol-xylol (1 to 3), xylol, and mounted in Canada balsam.

For brains of albino rats Craigie* found more satisfactory results with an earlier form of the method. Fixation was in Müller's fluid, which was renewed two or three times during the first week, and then left unchanged in the dark for two months or more. The tissue was then placed in distilled water for several hours; in 50 per cent alcohol for another three or four hours, in 70 per cent alcohol for twenty-four hours or more. It was imbedded in paraffin, by passing through 80 per cent, 95 per cent and absolute alcohol, cedar oil, into paraffin. The sections were affixed to the slide by the water-albumen method in the usual way, and when dry were passed through xylol and absolute alcohol, and then coated with a layer of very thin celloidin (0.5 per cent). This film hardens quickly in the air. The slides were then passed down through graded alcohols to water. Mordanting was effected by placing the slides upside down (supported on small pieces of glass) in a half-saturated solution of copper acetate, at about 35°C., overnight. After rinsing in distilled water they were stained for four to six hours, in lithium-hematoxylin (C. P.), the slides being horizontal with the sections underneath again. After washing they were differentiated with the borax-ferricyanide solution (D, p. 339). They were then washed in running water for twenty-four hours, dehydrated, cleared and mounted in Canada balsam.

3. Pal-Weigert Method, Modified. The following method is one used at the Netherlands Central Institute for Brain Research, Amsterdam, by Dr. Ariens Kappers, and is applicable both to human and comparative material.

As in other methods, fixation is in 10 per cent formalin, one week or longer. This is followed by immersion in 3 per cent potassium dichromate solution (primary mordant) for one to four weeks, according to the size of the blocks. The tissues are then washed well in running water, dehydrated in graded alcohols, imbedded in parlodion and cut at 25 μ to 75 μ. If the sections are being kept serially they should be converted into films at this stage on large slips. The individual sections or films are then placed for one to two hours in 100 c.c. of 0.5 per cent chromic acid to which has been added 1 c.c. of 1 per cent solution of osmic acid (secondary mordant). Rinse sections in water briefly, and stain twelve to twenty-four hours in the following hematoxylin stain (Kultschitsky's hematoxylin) freshly made up:

Ripened 10 per cent solution of hematoxylin in abs. alc.	10 c.c.
Acetic acid, glacial	1 c.c.
Distilled water	100 c.c.

This solution may be used either cold or warm. If kept in an incubator at not over 37°C. the color becomes very intense, but the drawback is that the sections tend to become brittle.

Wash in water until the color ceases to come out (not more than an hour is necessary) then begin the differentiating process. Immerse in 0.5 per cent potassium permanganate in water for thirty seconds, rinse in water, and pass into the following solution (Lustgarten's):

1 per cent potassium sulphite, in distilled water	100 c.c.
1 per cent oxalic acid, in distilled water	100 c.c.

*Craigie, E. H. The Finer Anatomy of the Central Nervous System of the Albino Rat. Toronto, 1925 (Method 126).

Mix the two solutions just prior to use. The differentiation must be watched closely, for it may proceed very rapidly, usually requiring not more than five minutes. The sections may be taken out before the differentiation is complete, and after washing briefly in water, place them again in the permanganate solution for thirty seconds, rinse and then place in the Lustgarten solution as before. By this process the background of gray matter is bleached out while the white matter stands out in strong contrast with its dark blue color. It is important not to leave too long in the permanganate solution, for the staining depends on the presence of potassium dichromate, and if this is removed it cannot be replaced, even by a renewed treatment with potassium dichromate. For this reason it is better to keep the sections in the differentiating fluids for a short time only, and to repeat the processes even several times, in order to control the reactions.

The sections, after being rinsed in tap water, are then transferred to distilled water, to which has been added a little lithium carbonate (water 500 c.c.; sat. aq. lith. carb., 1 c.c.) where they remain for twenty minutes; or they may be left overnight. Dehydrate in graded alcohols, clear in carbol-xylol followed by xylol and mount in balsam.

With the Pal method the differentiation between the myelinated fibers and the surrounding tissues may be carried to a greater degree than in the original methods of Weigert. For some purposes this is desirable, but for others it may not be an advantage. In the original Weigert the cell-bodies are brownish in color and in a general survey of a region, this adds to the completeness of the picture.

4. Iron Hematoxylin Myelin Stain. This method of staining myelin (Morgan),* is especially useful on old formalin-fixed material, which may be refractory to other methods, but is applicable also to fresh material following nearly any fixation. The directions are as follows:

1. Fix parts of central nervous system in formalin, alcohol-formalin, alcohol, Bouin's, Carnoy's, Flemming's, etc. Dehydrate, imbed in either paraffin or celloidin, and section.
2. Mordant sections two hours or less in 4 per cent aqueous solution of iron alum [ammonio-ferric sulphate, $(NH_4)_2Fe_2(SO_4)_4$].
3. Stain three to eight hours in well-ripened Heidenhain's hematoxylin (hematoxylin, 0.5 gm.; absolute alcohol, 10 c.c.; distilled water, 90 c.c.).
4. Destain in 2 per cent iron alum for a few minutes until the larger fiber tracts become well outlined.
5. Rinse in tap water.
6. Complete the differentiation in 0.5 per cent hydrochloric acid; in a few seconds the background becomes colorless and the fibers well differentiated.
7. Wash the sections in running water, for at least an hour, to remove the acid and alum.
8. (Counterstain with well-ripened neutral red, if desired.)
9. Dehydrate, clear in xylol and mount in balsam.

In successful preparations the myelinated fibers are stained a dark blue, while the background is clear and white. In destaining with the 2 per cent alum, the process should be stopped by placing the sections in tap water before all the blue of the hematoxylin has been extracted from the background; and the treatment with the hydrochloric acid should be very

* Morgan, Loc. cit. p. 326.

brief and should be carefully watched. When the proper stage has been reached the decolorizing is stopped by transferring the sections to tap water.

VII. Methods for Degenerating Myelin

1. Marchi Method. When a nerve-fiber is cut across or severely injured, the degeneration of the distal portion of the axon is accompanied by changes in chemical constitution and structural form of the myelin. The myelin loses its uniform distribution and becomes aggregated into little globules of varying sizes, along the neurokeratin framework. At the same time appears an acid, oleic acid, which stains readily with osmic acid. By using a solution containing potassium dichromate before, or at the same time as, the osmic acid a differential staining reaction on normal and degenerating myelin is obtained. The normal myelin is quickly oxidized by the potassium dichromate, and thus cannot be oxidized by the osmic acid. The normal myelin therefore remains unstained, while the fatty acid of the degenerating myelin is blackened.

Summary of Marchi Method.

1. Fix blocks of tissue (entire regions of small mammals) in Müller's fluid or 3 per cent potassium dichromate for three weeks, changing several times. The time required may vary from one to five weeks.

2. Cut out the parts desired, 2 to 3 mm. thick, and place directly in the following solution:

Müller's fluid, or 3 per cent pot. dichromate	2 parts
1 per cent osmic acid	1 part

in dark

Leave in this for one to three weeks, renewing the solution weekly.

3. Wash in running water for twenty-four hours.

4. Dehydrate and imbed in parlodion.

5. Cut sections 10μ to 50μ, according to purpose, and mount in balsam.

The degenerating myelin shows as black dots and flakes on a yellowish or brownish background of normal myelinated fibers, neuroglia and nerve-cell bodies.

As the process of degeneration is a progressive one, ending in total degeneration or regeneration, there is a limited time during which the myelin droplets are most numerous. Allen* considers this to be at the eleventh day after the injury in small mammals. Usually the material is taken between the tenth and the twenty-fifth day in mammals.

Besides the black particles of the degenerating myelin, an irregular precipitation may also occur, and it is necessary to be on one's guard in interpreting the appearances. The usual care must be taken in handling and removing the fresh tissues, lest the degenerating myelin be displaced. Allen, studying the degeneration of the tractus solitarius in the guinea pig, placed the entire brain, except the cerebral hemispheres, in 3 per cent potassium dichromate for four days, then cut it into blocks 5 to 6 mm.

* Allen, W. F. *J. Comp. Neurol.*, 1923, xxxv, 171.

thick, and returned them to the dichromate solution for another two weeks. For his second solution he used 3 parts of potassium dichromate to 1 part of the 1 per cent osmic acid, for three weeks. He dehydrated thoroughly and imbedded carefully in celloidin, in order to insure good sections. He found no disappearance of the stained myelin, because of the length of the imbedding process.

2. **Sudan III.** The usual fat stains, as sudan III, are also applicable to degenerating myelin.

VIII. Methods for Demonstrating Shape and Relationship of Neurons

1. **Golgi Silver Methods.** These consist essentially of immersing fresh pieces of nervous tissue first in a solution containing potassium dichromate (and usually osmic acid), and then in silver nitrate. A black deposit of a reduced silver salt is formed in and around the processes and cell-bodies of many of the neurons. Only a small fraction of the neurons and neuroglia are thus brought into view, one here and one there, but these are often shown in their entirety, so that the form of their cell-bodies and the distribution of their processes stand out prominently on a nearly colorless background. This selectiveness is a great advantage, for if all neurons were equally blackened the individual would be masked. In addition to the neurons, however, other structures, as blood vessels and connective tissue fibers may be blackened, and there is often an irregular distribution of black precipitate, especially in the outer portion of the block. As the method is largely empirical, results are uncertain, but usually something of interest is to be seen. Excellent examples of the results obtained by Golgi himself are contained in his collected works, "Opera Omnia."* With Golgi's own methods the best results are obtained with neonatal animals up to two to three weeks old, but the methods are applicable to all ages of material.

There are three main methods of Golgi himself, and a modification of one of the earlier methods by Cox, known as the Cox-Golgi method. The rapid method of Golgi and the Cox modification are the ones most frequently used on fresh animal tissues. The formalin modifications are useful for adult tissues, including human.

a. The Rapid Method of Golgi.

1. Fix and harden a number of thin pieces of brain or spinal cord, preferably not over 5 mm. thick, in the following solution (Golgi's fluid) for two to eight days:

Potassium dichromate, 3 per cent to 5 per cent aqueous	4 parts
Osmic acid, 1 per cent aqueous	1 part

This should be renewed if it becomes turbid. Have a thin layer of absorbent cotton on the bottom of the dish to allow free access of the fluid.

2. Take out a piece each day beginning with the second day, rinse in distilled water and dry the outside with filter paper.

* Golgi, C. Opera Omnia, 3 vols. (vols. I and II normal: vol. III pathological). Milano, 1903.

3. Place in 1 per cent aqueous solution of silver nitrate on absorbent cotton for one to two days. As a copious precipitate forms, change once or twice, using only small quantities of the silver nitrate, and then place in a larger quantity for the rest of the time. The material may be kept at room temperature or on an oven, not over 35°c., and it is usual but apparently not necessary to exclude the light.

4. Rinse in 80 per cent alcohol, several changes, to wash out the silver nitrate.

5. Thick sections are cut free-hand, or as frozen sections, or after rapid imbedding in parlodion or paraffin. The best results are obtained after rapid imbedding in parlodion.

Transfer to 95 per cent alcohol for one to four hours according to size; absolute alcohol, for the same length of time; absolute alcohol and ether, one hour; thin parlodion, in a loosely corked vial on the oven for several hours, until the parlodion thickens; block; harden in vapor of chloroform; cut thick sections in 95 per cent alcohol, and keep in this until cleared and mounted.

6. The sections may be mounted in the sandarac mixture of Cox, without a cover-glass, or in heated balsam, either with or without a cover-glass. In the Instituto Cajal the original formula of Cox* is still used for covering the Golgi and Cox-Golgi preparations, without the use of cover glasses. This consists of sandarac 75 gm., camphor 15 gm., turpentine 30 c.c., lavender oil 22.5 c.c., absolute alcohol 75 c.c., castor oil 5–10 drops. After the sections are placed on the slip, the 95 per cent alcohol is blotted off carefully and the sandarac mixture applied directly. This hardens quickly and several applications are made in the course of the day.

When mounted in balsam they must be heated; otherwise the preparations soon deteriorate. After the sections are thoroughly dehydrated and cleared, in cedar oil, in creosote followed by xylol, in oleum origani cretici and xylol or in bergamot oil and xylol, there are two methods of mounting them:

a. Cover the sections on the slide with a relatively large quantity of thick xylol balsam, and heat carefully over a flame or on a hot plate to drive off the solvent, so that the balsam becomes hard as soon as it cools. Just before the balsam cools, a warm cover glass may be placed on the preparation and gently pressed down.

b. Melt some hard balsam in a porcelain crucible. Warm a slip, so that it is not too hot to hold, place the sections on it, and cover with a drop of the fluid balsam. It hardens immediately, as it cools. A cover glass may be added as before.

The duration of the hardening process in the dichromate-osmic solution (1) is of great importance. This varies with the kind of impregnation aimed at, the region of the nervous system, age of the individual, whether embryonic or adult, species, temperature at which the hardening takes place, and quantity of hardening fluid. The times for the spinal cord are given as two to three days to show neuroglia, three to five days for nerve-cells, five to seven days for nerve-fibers and collaterals, but these are true only in a very general way, for neuroglia and nerve-fibers usually appear irrespective of the duration in the hardening fluid.

When the quantity of the tissues is greater the fluid is relatively less effective, and the hardening process is slowed. When there is under-hardening there appears only a diffuse red precipitate of silver chromate. When there is over-hardening there is absence of impregnation, and only sharply defined crystals. The preparations are best when there are fewer elements impregnated, but these are shown with great clearness. As to age, generally the older the tissue, the longer the time required in the hardening fluid.

* Cox, W. H., *Arch. f. Mikr. Anat.*, 1891, xxxvii, 16.

For an eight-month human fetus, Strong* found the six-day period the best. Fetal and newborn human tissues, under favorable conditions, react for twenty-four hours after death.

The double impregnation process, suggested by Cajal, often improves the results.

A sample procedure with pieces of cerebelleum of a fifteen-day or thirty-day-old cat or dog is as follows.

Five or six flat pieces, not over 4 mm. thick are placed in 30 c.c. of Golgi's osmic-dichromate fluid for three days on the oven at 20° to 25°C., in a glass-stoppered bottle. A stock of this fluid may be kept in a brown bottle in the dark. Place the pieces directly in a small quantity of 0.75 per cent silver nitrate, where there is a copious precipitate; then into 100 c.c. of 0.75 per cent silver nitrate, for thirty hours, on the table. Dry the outside of the pieces by turning them over on filter paper, and place them in the original 30 c.c. of osmic-dichromate fluid, and leave for one day on the oven. Change the pieces directly into the original 100 c.c. of 0.75 per cent silver nitrate, and leave for one day.

After rinsing off the silver in water, dry the outside of the pieces on filter paper. Dip them into 95 per cent alcohol for one to two minutes, and dry on filter paper. Mount directly on cubes of hard paraffin, by heating one surface of the latter with a heated spatula until the paraffin begins to melt. Place a piece of tissue on the melted surface, and apply a hot needle to paraffin around the base of the piece of tissue, to ensure good contact. Dip in 95 per cent alcohol. Cut sections 85 to 100 μ. Wash sections well in 95 per cent alcohol, using 5 to 6 changes, total time not more than a half hour. Transfer sections to slip, press gently and dry with filter paper. Drop on Cox's sandarac mixture (see above). When firm, repeat application once or twice, until there is a permanently smooth surface, and protect from dust and light. Such preparations have been kept for twenty years and more without deterioration of the impregnation.

Windle,† in following the entering nerve-fibers of the trigeminal nerve within the brain stem, found the best results with this procedure:

Brains of large fetal pigs were placed in 3.5 per cent potassium dichromate for forty-eight hours; in the osmic-dichromate mixture (1 per cent osmic acid, 15 c.c., 3.5 per cent potassium dichromate, 85 c.c.) for seventy-two hours; again in 3.5 per cent potassium dichromate for five to six days; then into 0.75 per cent silver nitrate, for seven to ten days, at room temperature in the dark. The brains were halved, and placed in 95 per cent alcohol, imbedded in celloidin, and cut at 150 μ. Abundant time was allowed for complete infiltration by the celloidin, without affecting the silver deposit.

b. Modifications of Golgi Methods with Formalin. Strong,‡ Kopsch§ and others have suggested useful modifications in which formalin is substituted for the osmic acid in the hardening fluid. These react better with adult than with young tissues, and may be used on human autopsy material.

Strong's formula for the hardening fluid is:

Potassium dichromate, 3½ to 5 per cent	100 c.c.
Formalin, full strength	2½ to 5 c.c.

* Strong, O. S. Golgi silver methods. In M. Allen Starr's Atlas of Nerve Cells. 1896.
† Windle, W. F. *J. Comp. Neurol.*, 1926, xl, 229.
‡ Strong, O. S. *Anat. Anz.*, 1895, x, 494.
§ Kopsch, F. *Anat. Anz.*, 1896, xi, 727.

Use a number of thin flat pieces, and each day, for five to eight days, transfer a piece to 1 per cent silver nitrate, and proceed as in the original method. As the formalin penetrates better than the osmic acid, the stage of the hardening process which is favorable for impregnation may be reached as early as eighteen hours. It is found, also, that this favorable stage lasts longer than with the osmic acid mixture, and consequently good results are more certain over a greater range of time.

In Kopsch's modification, the tissues are first placed in a mixture of 3.5 per cent potassium dichromate, 4 parts, and formalin, 1 part, for twenty-four hours, and then in 3.5 per cent potassium dichromate alone for three to six days, before being silvered. The Purkinje cell dendrites and other structures of the cerebellar cortex are often well shown in human autopsy material by this method.

Brookover* fixed adult brains of Amia in 10 per cent formalin neutralized with lithium carbonate or ammonium, for five to twelve hours, and followed this with the regular rapid method. This treatment seemed to favor the impregnation of axons.

c. Cox-Golgi Method. This is one of the simplest procedures for demonstrating the relations of dendrites and axons to the cell-body. It gives excellent preparations of the pyramidal cells of the cerebral cortex of the adult albino rat after three weeks' impregnation.

To Prepare Cox-Golgi Fluid. There are three stock solutions of salts which keep indefinitely.

A. 5 per cent aqueous solution of potassium chromate.
B. 5 per cent aqueous solution of potassium dichromate.
C. 5 per cent aqueous solution of mercuric chloride.
Dilute 16 c.c. of A with 40 c.c. of distilled water.
To 20 c.c. of B add 20 c.c. of C.
Mix these two solutions and allow to warm on incubator.

This quantity is suitable for the brain of an albino rat. Place absorbent cotton on the bottom of the bottle, before putting in the brain, so that the tissues do not rest on the glass. To prevent evaporation, seal around the cork with paraffin, and leave on incubator for about a month. Mann† advises changing the solution after the first and the third day. At the end of several weeks the tissue becomes brownish in color and rather friable. It may be tested by slicing off portions with a safety razor blade or making frozen sections. To obtain good sections, it is best to imbed in parlodion in the usual way. As the sections are to be cut thick, they may be imbedded rapidly. This is done by placing the tissue in a loosely-corked vial of thick parlodion (after one day in thin parlodion) on top of an oven. The parlodion thickens rapidly and the tissue may be blocked at the end of the day. The sections should be well dehydrated and cleared before mounting in damar

* Brookover, C. *J. Comp. Neurol.*, 1910, xx, 49.
† Mann, Gustav. Physiological Histology. N. Y., 1902, p. 272.

or balsam. They may also be mounted like other Golgi preparations in hard balsam or in the sandarac mixture of Cox, without cover glasses, if one wishes to keep them a long time.

The nerve-cells and processes are outlined in black on a light yellowish or colorless background. There are also irregular black deposits and some blood vessels may be impregnated, but in both these respects the Cox method is often better than the Golgi silver methods. Preparations mounted under cover glasses usually deteriorate in several years. Bremer* found that after thorough imbedding (usual steps of dehydration, followed by one week or more in thin celloidin, and an equal time in thick celloidin) the sections may be easily stained with hematoxylin and eosin, without injuring the impregnation, and that these stained specimens showed less of the mercury deposit after several months than did the specimens without stain.

2. Vom Rath's Platinic Chloride Method. Of the several methods of vom Rath, the following gives good results with growing processes of neuroblasts and neurofibrils. This is the picric-acetic-osmic-platinic chloride method, as used by Neal† on young selachian material.

1. Fix for one to three days in the dark in the following mixture, changed daily:

Picric acid, sat. aq. sol., filtered	200 c.c.
Glacial acetic acid	2 c.c.
10 per cent platinic chloride in distilled water	10 c.c.
2 per cent osmic acid	25 c.c.

This makes embryos brittle, and in order to avoid injuring them, the fluid should be changed by pipette, without moving the embryos from the dish.

2. Transfer to 0.5 per cent pyrogallic acid, in the dark, for twenty-four to forty-eight hours, changing several times.

3. Dehydrate in graded alcohols, preferably by slow continuous change of fluids; clear in xylol; imbed in paraffin by adding the paraffin to the xylol; block in hard paraffin; section at four to eight μ.

Norris and Hughes‡ in tracing out the nerve components of cerebrospinal nerves in young elasmobranchs, e. g., *Squalus* (10 to 15 cm. long), used a preliminary fixation in 10 per cent formalin before placing the entire heads in the vom Rath fluid. This allowed the use of heads 20 to 30 mm. long, and prevented the brittleness to a great extent. The material was imbedded in celloidin and cut serially at 15 μ to 20 μ. In these preparations only nerves and muscles were blackened, the background being nearly colorless.

IX. Methods Mainly for Peripheral Nervous System

1. Methods for Isolating Nerve Fibers. To examine nerve fibers in the fresh condition a small piece of nerve just taken from the body is placed

* Bremer, J. L. *Anat. Record*, 1910, iv, 263.

† Neal, H. V. *J. Morphol.*, 1914, xxv, 1.

‡ Norris, H. W. and Hughes, S. P. *J. Comp. Neurol.*, 1919, xxxi, 293.

on the slip, and one end of the piece is separated into many little strands by pulling it apart with sharp-pointed needles, and by stroking the little bundles lengthwise with the point of the needle. The use of a dissecting microscope for these manipulations is very helpful. To examine with a microscope merely add a drop of physiological salt solution and cover. The myelin readily changes its arrangement, forming irregular globules and droplets of varying sizes. This occurs on the addition of water, on pressure, and as a post-mortem change in the body.

To fix nerves intended for the isolation of individual fibers, 1 per cent osmic acid or 10 per cent formalin may be used. The former is best for the demonstration of nodes of Ranvier, the latter is good for the arrangement of the neurokeratin. A convenient stain for the latter is dilute hematoxylin (1 part hematoxylin with 20 parts of distilled water) for some hours or overnight. This usually stains the sheath nuclei well. To show especially the neurokeratin, the fresh nerves may be digested with trypsin or pepsin; or fixed in 95 per cent alcohol to dissolve the myelin, and stained with dilute hematoxylin and acid fuchsin. To make permanent mounts of isolated nerve-fibers, small pieces (not over 1 mm. in diameter) are stained, dehydrated in alcohols, cleared in clove oil, where they are carefully teased out, and mounted in balsam. In fixed material the connective tissue framework is tougher than in the fresh condition but the nerve fibers are likewise more resistant. The large myelinated fibers are easiest to isolate; the unmyelinated fibers require more careful teasing.

2. Methods for Unmyelinated Nerve Fibers. *a. Ranson's Pyridine Silver Method.** This is a modification of the Cajal reduced silver method, and gives an excellent differential coloration of unmyelinated fibers, both in nerve-trunks and in ganglia.

1. Fix in absolute alcohol, to which has been added 1 per cent of concentrated ammonia, for forty-eight hours.
2. Rinse in distilled water one-half to three minutes according to the size of the blocks.
3. Transfer to pyridine twenty-four hours.
4. Wash in many changes of distilled water twenty-four hours.
5. Place in the dark three days in 2 per cent aqueous solution of silver nitrate, at 35°C.
6. Rinse with distilled water.
7. Reduce in the following, twenty-four to forty-eight hours:

Pyrogallol	4 gm.
5 per cent formalin in distilled water	100 c.c.

8. Rinse in distilled water, dehydrate, clear, imbed in paraffin. The sections, after mounting, are ready for examination.

The unmyelinated fibers are black, and are sharply differentiated from the surrounding light yellow endoneurium; myelinated fibers are stained

* Ranson, S. W. *Rev. Neurol. & Psychiat.*, 1914, xii, 467.

yellow, and are surrounded by a colorless ring of myelin. Ranson has used it for studying the myelinated and unmyelinated components of spinal nerves, and of the vagus nerve in several species, as well as spinal ganglia, sympathetic trunk and ganglia, and spinal cord.

Koch,* studying the cranial nerves especially for the presence of unmyelinated fibers, used the above method and also the following: the dissected specimens were laid on glass slips and fixed in 50 per cent pyridine for seven days. They were then washed, silvered, washed, reduced, etc., as in the above method.

Ranson and Billingsley† found that in dealing with small nerves and ganglia, the method failed to give good results, apparently because the volume of the tissue was too small. This was overcome by coating the nerve with other nervous tissue in the following manner: a fine silk thread was tied to the nerve, and by means of a long fine needle it was drawn into the lateral half of the spinal cord along the line of the ventral gray column. After fixation two hours in the ammoniated alcohol the block was pared down with a razor into the form of a bar, not over 4 sq. mm. in cross-area. The cord was dissected away from the nerve just before it was dehydrated and cleared in preparation for imbedding.

This method is also useful for showing the degeneration of unmyelinated fibers, as employed by Ranson and others. Thus Johnson,‡ in an experimental study of the degeneration of the extrinsic nerves of the small intestine by this method found the following procedure important in fixing the tissues: Physiological salt solution was injected through the mesenteric circulation and through the lumen of the selected length of the intestine until it was blanched. Then the ammoniated alcohol mixture was introduced by the same routes under slight pressure. The intestine was thus kept distended for an hour, when strips, 2 mm. or less in diameter, were cut off along the mesenteric attachment, threaded through pieces of spinal cord, and the whole returned to fresh fixing fluid to complete the fixation.

Modification of Ranson's Method for Calcified Tissues. For material requiring decalcification Huber and Guild,§ in a study on the peripheral distribution of the nervus terminalis, adapted the method as follows:

1. Adult or young animals and embryos of sufficient size to admit of injection are perfused with the ammoniated alcohol solution. The ganglia, nerve-trunks and pieces of the central nervous system as desired are then removed and placed in a similar ammoniated alcohol, in which they remain two to four days, depending on the size of the tissue mass to be fixed.
2. Transfer to distilled water; leave until the pieces sink.
3. Decalcify in 7 per cent nitric acid, made up in distilled water. Test from day to day.
4. Wash in distilled water for about one-half hour, changing the water frequently.

* Koch, S. L. *J. Comp. Neurol.*, 1916, xxvi, 541.
† Ranson, S. W. and Billingsley, P. R. *J. Comp. Neurol.*, 1918, xxix, 313.
‡ Johnson, S. E. *J. Comp. Neurol.*, 1925, xxxviii, 299.
§ Huber, G. C. and Guild, S. R. *Anat. Record*, 1913, vii, 253.

5. The pieces are then transferred to alcohols of 80, 90 and 95 per cent, to which is added 1 per cent of concentrated ammonia. A thorough treatment with ammoniated alcohol at this step seems essential; three to eight days, depending on the size of the pieces, are required.

6. Rinse in distilled water and place in pyridine for twenty-four hours.

7. Wash thoroughly in distilled water, the water being changed frequently. The transfer from pyridine to water is made preferably through several mixtures of the two, containing increasing percentages of water.

8. Transfer to 2 per cent solution of silver nitrate in distilled water, three to five days in the dark at a temperature of about 35°C.

9. Rinse in distilled water and place for one to two days in a 4 per cent solution of pyrogallic acid in 5 per cent formalin.

10. Dehydrate thoroughly, beginning with 80 per cent alcohol. Clear in xylol and imbed in paraffin. A prolonged stay in the warm oven even to forty-eight hours, to insure thorough paraffin penetration, does not seem to affect the stain.

It was found possible to stain half the head of a six-day rabbit, head and neck of medium-sized frog, head of a small turtle, etc. Huber and Guild consider that the method of fixation by preliminary injection of the ammoniated alcohol is advantageous in preventing to a great extent shrinkage and distortion of the peripheral layers of cells in ganglia, and the impregnation and reduction of the silver seems more uniform and more certain. The procedure for perfusing is as follows: The animal is chloroformed and the heart incised at once, in order to drain the vascular system as completely as possible. A cannula is inserted into the heart or aorta, or if possible into the artery to the part desired. The cannula is filled with physiological salt solution, and connected with the ammoniated alcohol container. The ammoniated alcohol is then rapidly injected under a pressure of 5 to 10 pounds, this being continued until the parts seem well injected. The skin and overlying tissues of the brain and nerves are then removed and the specimen placed entire in a large quantity of the ammoniated alcohol. Further cutting of the pieces is delayed until after decalcification and the second ammoniated alcohol treatment. It is essential to have fresh pure chemicals, absolutely clean utensils and fairly constant temperature.

Rhinehart,* in studying the peripheral distribution of the nervus facialis in the albino mouse, used this method on whole and half heads of animals up to twenty-three days of age. He used the double imbedding method and cut serially at 15 μ. The main trunks and branches of the nerves, the nerve-fibers in muscles and connective tissue, ganglion cells on the roots of the cranial nerves, and the cells and fibers of the central nervous system were well impregnated for his purpose. With slight variations this method was also used by Johnson† for the lateral line sense organs of *Squalus*, by Brookover‡ and Larsell§ for the peripheral distribution of the nervus

* Rhinehart, D. A. *J. Comp. Neurol.*, 1918, xxx, 81.

† Johnson, S. E. *J. Comp. Neurol.*, 1917, xxviii, 1.

‡ Brookover, C. *J. Comp. Neurol.*, 1917, xxviii, 349.

§ Larsell, O. *J. Comp, Neurol.*, 1918, xxx, 1.

terminalis, by Stewart* in studying the development of the cranial sympathetic ganglia of the fetal albino rat, and by Gurdjian† for the olfactory connections in the adult of the albino rat.

b. Osmic Acid Method for Myelinated Nerve-fibers. Osmic acid is the classical substance for demonstrating myelinated nerve-fibers, and is still one of the best. The myelin is blackened by the osmic acid, and the osmicated myelin is resistant to the procedures of imbedding in paraffin or parlodion.

The osmic acid is used in ½ of 1 per cent solution, or at half this strength. As osmic acid dissolves slowly, the stock solution of 2 per cent or 1 per cent should be prepared beforehand. In preparing the stock solution wash and dry the outside of the sealed glass tube in which the osmic acid crystals are bought. File around the middle of the tube and holding it within the folds of a clean towel, break it into two parts. Drop the pieces of the glass containing the osmic crystals into the appropriate quantity of distilled water in a glass stoppered bottle. If there is no contamination by organic substances, and if the solution is kept in a dark place, not exposed to high temperatures, it remains unreduced for several months. After some time, it becomes brownish in color, a black precipitate settles on the bottom of the bottle, and the solution is no longer useful.

In preparing material by this method it is well to use thin nerves, 1 to 2 mm. in diameter, as the osmic acid does not penetrate far. Expose the length of the nerve desired without handling or stretching it. To keep the nerve of the same shape and length, place a narrow strip of paper (or a match-stick or a glass rod) against the nerve. Cut the nerve at the ends of the part to be taken and remove this part by raising one end of the paper and cutting the connective tissue attachments. When freed drop the paper and nerve, the latter downwards, on the osmic acid. It soon begins to darken but fixation should be continued twenty-four hours. Immersion in the osmic acid may be prolonged to a week but the nerve becomes brittle. The material is then washed in running water for twelve to twenty-four hours (small pieces one-half hour).

When the nerve-fibers are to be examined whole they are placed, after washing, in glycerin and are ready to be torn apart. If mounted in glycerin and the coverslip cemented around the edge, they remain unaltered for years. Or, after washing, small bundles of fibers may be dehydrated by passing them directly into 95 per cent alcohol (changed twice, two to three minutes in each) and then cleared in clove oil for three to five minutes. They may be further torn apart, and then mounted in balsam under a cover glass.

In a study of the length of internodes, Takahashi‡ used the following procedure in isolating the fibers of the sciatic nerve of frogs:

* Stewart, F. W. *J. Comp. Neurol.*, 1919, xxxi, 163.

† Gurdjian, E. S. *J. Comp. Neurol.*, 1925, xxxviii, 127.

‡ Takahashi, K. *J. Comp. Neurol.*, 1908, xviii, 167.

A short piece of the fresh nerve, on a strip of cardboard, was fixed and at the same time macerated by leaving it twenty-four hours in the following solution:

Solution A	Osmic acid, 1 per cent	5 parts
	Chromic acid, 0.25 per cent	3 parts
	Hydrochloric acid 0.10 per cent	2 parts

Wash in running water twenty-four hours.
Transfer to solution B twenty-four hours.

Solution B	Glycerin	10 parts
	50 per cent alcohol	20 parts
	Hydrochloric acid	0.09 parts

After this the specimens are preserved in solution C.

Solution C	Glycerin	10 parts
	50 per cent alcohol	20 parts

This is renewed once or twice at intervals of twenty-four hours.

When the nerve-fibers are to be sectioned the material is cut into shorter lengths, dehydrated in graded alcohols, cleared in xylol and imbedded in paraffin. This method has been followed in studying the growth in diameter of myelinated nerve-fibers of the albino rat at different ages by Nittono,* and Donaldson and Nagasaka (p. 324).

In osmic acid preparations of nerves, between the blackened fibers are also to be seen many thin unmyelinated fibers. These are grayish in appearance, nearly colorless.

3. **Methods for Nerve Endings.** *a. Gold Chloride Method of Ranvier.* For many years gold chloride has been used to demonstrate the motor nerve-endings in striated muscle, the myenteric plexus of the intestine, the nerves of the cornea, etc. To demonstrate motor nerve-endings, use short muscles, as the intercostals of mouse, young rat, kitten or guinea pig. Cut out pieces 4 to 5 mm. broad, and place in fresh lemon juice filtered through muslin or filter paper. Leave here until the pieces become translucent, ten to twenty minutes.

Dip the pieces in distilled water and then place them in a 1 per cent solution of gold chloride, where they remain fifteen to twenty-five minutes.

The pieces are again passed through distilled water and placed in a bottle containing a 20 per cent solution of formic acid. This is kept in the dark for twenty-four to forty-eight hours. To find whether the impregnation has been successful take out a small piece on a slip in a drop of glycerin. The muscle fibers are usually reddish or purple, while the nerves and their endings are black. Sometimes the nerves show well even to their smallest branches, but the endings are indistinct. One cannot be sure that the preparation is unsuccessful until all parts have been examined, for usually at one region in a block the conditions have been right for a success-

* Nittono, K. *J. Comp. Neurol.*, 1920, xxxii, 231.

ful impregnation. The preparations are mounted in glycerin, and the cover glass cemented around the edge.

The procedure for the myenteric plexus of the stomach or intestine is the same as outlined above for the motor end-plates.

According to Miller* citric acid may be substituted for lemon juice with equally good or even better results. The thin strips of intercostal muscle of rabbit are placed in a 4 to 100 solution of citric acid in distilled water in the dark for twenty to thirty minutes. After rinsing in distilled water transfer to 1 per cent gold chloride in distilled water and keep in the dark for twenty to thirty minutes. Then place in a 33 per cent solution of formic acid, in the dark, for forty-eight hours. Rinse in distilled water and preserve in pure glycerin.

Garven,† studying the question of the sympathetic innervation of striated muscle, employed the modification of Ranvier's method as used in Golgi's laboratory:

1. Immerse small pieces in 25 per cent aqueous solution of pure formic acid, for ten to fifteen minutes. Use only enough solution for the complete immersion of the pieces. The pieces should be teased a little in the solution.
2. Take pieces from the acid solution and place on a clean folded towel or duster, cover the pieces with another fold of the towel, and press gently to absorb as much acid as possible.
3. Transfer to 1 per cent solution of gold chloride, just sufficient to cover the pieces, for twenty minutes at most, shaking the pieces around in the dish several times. Keep dish covered with blue or yellow glass. No iron instruments must be allowed to come in contact with this or the subsequent baths; use either bone-pointed forceps or paraffin-coated points of metal ones.
4. Mop as per instructions in 2.
5. Transfer to 25 per cent formic acid, using just enough to cover the pieces, and keep in absolute darkness for twenty-four hours.
6. Mop as per instructions in 2.
7. Preserve in pure glycerin.

Nerve-fibers are intensely purple or black. Good preparations keep five years or more, becoming clearer with time. Cleanliness as to dishes and instruments is important, and solutions are made with double or treble distilled water.

b. Intra Vitam Methylene Blue Method. This was introduced by Ehrlich‡ and has been used by many investigators, notably by Dogiel,§ who has fully described the procedures. A useful account of the method has also been given by Wilson.‖

* Miller, C. H. *Anat. Record*, 1923, xxv, 77.

† Garven, H. S. D. *Brain*, 1925, xlviii, 380.

‡ Ehrlich, P. Das Sauerstoffbedürfnis des Organismus, eine farbenanalytische Studie. Berl., 1885.

§ Dogiel, A. S. Methylenblau zur Nervenfärbung, in Encylopädie der mikroskop. Technik. Ed. 2, 1910; Ed. 3, 1927.

‖ Wilson, J. G. *Anat. Record*, 1910, iv, 267.

The diluted stain is applied directly to the fresh tissues. This may be done in several ways: (1) by moistening thin pieces of the excised tissue with the fluid, (2) by perfusing the blood vessels of the whole animal or of the region desired, (3) by injecting into the natural cavities of the body, as into the peritoneal cavity, into the pleural cavity or into the lungs through the trachea, (4) by injecting into the loose connective tissues of the part to be examined.

The dilution of the staining fluid varies with the material and the purposes of the study. Thus Cole,* studying the myenteric plexus of the frog's intestine, used a dilution of 1:10,000; Larsell† for nerve termination in the lung of the rabbit, and Langworthy‡ for proprioceptive nerve-endings in the tongue of fetal pigs, used 1:2,000; while Johnson,§ studying sympathetic ganglia of the gangliated cord of the frog, used a dilution of 1:25.

The time required for the staining is usually between fifteen and thirty minutes but may be longer, and has to be determined by trial.

After the nerves are stained blue the tissues may be studied at once, but as the stain is transitory it is preferable to fix it so that the tissue may be imbedded and sectioned. Ammonium molybdate is used for the fixation of the stain in the tissue, using an 8 per cent solution in physiological salt solution or Ringer's solution, for one-half hour to overnight. The tissue is then washed in running water for an hour and dehydrated rapidly, in order to avoid losing the color. From the water the tissue is passed into 95 per cent alcohol for ten to sixty minutes, and then into absolute alcohol for one to two hours. If possible have the alcohols cold and keep them in the refrigerator, at several degrees above freezing, during the dehydration of the tissues. The tissues are then cleared in xylol, and if in the form of thin membranes or small shreds, they may be mounted at once in balsam.

If they are of larger size they are imbedded in paraffin and sectioned. The sections may be lightly counterstained.

In successful preparations the ganglion cells with their processes, myelinated and unmyelinated, and their end-arborizations are colored blue. Other tissues should be nearly colorless unless injured by pressing or cutting, in which condition they become deeply colored. Elastic fibers and connective tissue cells may assume a blue color, and may be then mistaken for nerve elements if the latter are not in evidence.

The peculiarity of the reaction of the dye in this method is that the maximum staining lasts only a short time in the fresh tissue. This is explained by the fact that the blue compound becomes colorless when the oxygen content of the tissue becomes lessened. For this reason, when in a

* Cole, E. C. *J. Comp. Neurol.*, 1925, xxxviii, 375.
† Larsell, O. *J. Comp. Neurol.*, 1921, xxxiii, 105.
‡ Langworthy, O. R. *J. Comp. Neurol.*, 1924, xxxvi, 273.
§ Johnson, S. E. *J. Comp. Neurol.*, 1918, xxix, 385.

fresh preparation the color is seen to be fading, the color may sometimes be restored by removing the cover glass and allowing the oxygen of the air to have access to the tissue.

(1) Summary of Method by Local Application.

1. Place thin pieces of fresh tissue on a slip or in a shallow dish (the bottom of the latter may be covered with a thin layer of glass-wool or feathers on which the tissues rest) moistened with the staining solution, e. g., 1 part of methylene blue, *intra vitam*, to 1000 to 2000 parts of physiological salt solution.

2. Drop on enough staining fluid from time to time to keep the tissues moist, with a film of stain over them.

3. After fifteen minutes the tissues are examined with low magnification and at short intervals thereafter until the nerves are colored blue. The length of time varies, but if conditions are favorable it should not be more than an hour, or at most two hours.

4. Fix the stain by placing the tissues in the 8 per cent ammonium molybdate solution made up with physiological salt solution or Ringer's fluid.

5. After washing and dehydration the tissues may be either cleared in xylol and mounted in balsam or imbedded in paraffin and sectioned.

A variation of this was used by Cole (p. 355) who immersed the entire digestive tube of the frog in a 1 : 1000 solution for one hour. Small segments were then cut out, flattened between two slips and examined under low power. If the stain was not satisfactory the tissues were replaced in the stain; if satisfactory, they were fixed in 8 per cent molybdate in Ringer's, for a half hour. The tissues were placed in a muslin bag and washed for an hour. The excess water was squeezed out on a towel and the tissues placed in ice-cold 95 per cent alcohol for ten minutes, then in ice-cold absolute alcohol for ten minutes. They were cleared in xylol, if thin, or in cedar oil if thick, and mounted in balsam.

(2) Summary of Method by Injection.

1. Insert a cannula into the heart or aorta, or into the main artery of the part to be examined. Inject the filtered dilute solution of the dye (for mammalian tissues 1 to 10,000 or even more dilute) until the part has a distinctly light blue color.

2. Leave undisturbed for a quarter of an hour, after which time thin pieces or slices of the tissue are removed and placed on a slip or in a dish moistened with the dye.

3. These are examined under a low power of the microscope, without cover glass every two or three minutes until the nerve-cells, nerve-fibers or nerve-endings seem satisfactorily stained.

4. Fix the stain in 8 per cent ammonium molybdate, one-half hour to overnight, and continue as in the preceding summary.

In the case of large fetuses, as of pig, the injection may be made through one of the umbilical arteries. Langworthy (p. 355) used this method, after first washing out the blood with physiological salt solution. For the dye he used 1 : 2000 methylene blue (after Ehrlich), rendered slightly alkaline with ammonium hydroxide. The tongue was removed and thin slices cut with a sharp knife. The sections were spread out on slips and placed in an

observation warm-box (as used for examining tissue cultures) until the nerve-endings appeared. The musculature of the tongue had to be separated in order to admit of free oxidation, and even then there were always large areas where the stain was not oxidized.

For the study of the nerve-endings in the lungs of rabbits, Larsell (p. 355) used warm 1:2000 methylene blue in Locke's solution, or in 0.9 per cent NaCl solution, and filled the lungs through the trachea, or injected the fluid through the pulmonary vessels. The lungs were allowed to remain undisturbed in the thorax for ten minutes. The excess of fluid was allowed to run out of the lungs in the cases in which they had been filled. Then, after both methods of application of the stain, the lungs were alternately inflated and deflated with air through the trachea, at the rate of 12 to 15 times per minute. It was found that twenty to twenty-five minutes was the optimum time for continuing the oxidation of the stain in the lung tissue in this manner.

The lungs were extirpated and filled with 8 per cent cold ammonium molybdate to which had been added 2 to 5 drops of 1 per cent osmic acid per 100c.c. of ammonium molybdate. They were immersed in a solution of the same composition and left in it overnight in the cold. After being washed in running tap water for one hour the lungs were filled with 95 per cent alcohol and immersed in 95 per cent alcohol for an hour, the alcohol within the lungs being changed several times during the hour. They were next placed in absolute alcohol for several hours to overnight, to complete the dehydration. Suitable pieces were cut out, 2 to 4 mm. thick, cleared in xylol, imbedded in paraffin, and sectioned at 25 μ to 100 μ thick. He found that the lungs injected through the pulmonary vessels showed the bronchial endings better, and that those injected through the trachea showed the endings in the walls of the pulmonary vessels more clearly.

The method of injection through the blood vessels, for the study of the sympathetic ganglia in the frog, was used by Johnson (p. 355) following the methods of Huber.* The latter found that, in a general way, the stronger solutions stained more readily the cell-body and the branches of the sympathetic neurons, while the weaker solutions brought to view more clearly the pericellular plexuses; there being, however, many exceptions. Huber used 1 to 4 per cent of Grübler's methylene blue, "rectificiert nach Ehrlich." Johnson injected the frogs through the ventral vein, using 3 to 4 per cent methylene blue in Ringer-Locke solution.

It is evident that the composition of the dye is very important in attaining good results. It is believed that the purer the methylene blue is, the more successfully it can be used for vital staining.

The method is applicable much more to the staining of peripheral ganglia (spinal, cerebral, sympathetic, parasympathetic), peripheral nerves and nerve-endings than to the staining of the elements of the

* Huber, G. C. *J. Morphol.*, 1900, xvi, 27.

central nervous system, although the latter may also be stained by this method.

(*c*) *The Sihler-Gad Method.* This is a useful method for nerve-endings in striated muscle and in the walls of blood-vessels (Gad).*

1. Fix thin pieces in the following solution, for eighteen hours:

Acetic acid	10 c.c.
Glycerin	10 c.c.
1 per cent solution of chloral hydrate	60 c.c.

2. Transfer to pure glycerin, where they are teased apart somewhat, and leave for one to two hours.

3. Stain in the following mixture, for three to ten days:

Ehrlich's hematoxylin	10 c.c.
Glycerin	10 c.c.
1 per cent solution of chloral hydrate	60 c.c.

4. Transfer to pure glycerin; change several times. The tissues are now ready for differentiation, but may remain in the glycerin without detriment for some time.

5. Differentiate in the solution used for fixation (1 above). The nerves and nerve-endings are shown deeply stained, somewhat purplish in color, while the muscle and other surrounding tissues are pale. For rat intercostals, more than twenty-four hours are required.

The methods of Cajal and Bielschowsky, already described, are also valuable for the study of nerve endings.

* Gad, J. *Arch. f. Anat.* Physiol. Abth., 1895.

NEUROGLIA AND MICROGLIA (THE METALLIC METHODS)

WILDER PENFIELD AND WILLIAM CONE

INTRODUCTION 359. Classification of interstitial cells of central nervous system 359. An outline for routine study of interstitial cells 365. NEUROGLIA ASTROCYTES (MACROGLIA) 366. Cajal's gold chloride sublimate method 367. General observations on silver staining 372. Del Rio-Hortega's silver carbonate method for astrocytes 374. Achúcarro's tannin-silver method 376. OLIGODENDROGLIA 378. Del Rio-Hortega's silver carbonate method for oligodendroglia 378. MICROGLIA 380. Del Rio-Hortega's silver carbonate method for microglia 380. SPECIAL METHODS 383. SOLUTIONS 386.

A. INTRODUCTION

A word of description may be given here because of the recent additions to our knowledge of the interstitial cells.* Like most real advances this added knowledge has resulted in simplification of some problems which formerly seemed complex. The origin of compound granular corpuscles, rod cells, ameboid glia, the so-called preameboid cells, etc. is now clear, making the pathology of the nervous system a much less bewildering field of endeavor than before. The investigation of tumors arising in the brain has also received fresh impetus.†

I. Classification of the Interstitial Cells of the Central Nervous System

		Normal Forms	*Pathological Forms*
Neuroglia	Astrocytes	a. Protoplasmic (gray matter)	Ameboid glia
		b. Fibrous (white matter)	Fibrous gliosis
			Giant glia
	Oligodendroglia	a. Perineuronal satellites	Acute swelling
		b. Interfascicular	Satellitosis
Microglia		Ubiquitous	Rod cells
			Compound granular corpuscles. (Gitterzellen)

1. Astrocytes (classical neuroglia, macroglia). The most complete description of these cells is that made by Cajal in 1913‡ after devising the gold chloride method. The astrocytes are star-shaped cells whose expansions radiate through the nervous tissue in all directions. They all possess one or more perivascular expansions which are applied to the surface of small vessels. They possess a granular cytoplasm which contains gliosomes, small granules said to be secretion granules.

Protoplasmic astrocytes (Fig. 1) are found in the gray matter. They are in general somewhat smaller than fibrous astrocytes, contain more glio-

* Penfield, W. Cowdry's Special Cytology, N. Y., 1928.

† Bailey, P. and Cushing, H. Tumors of the Glioma Group. Phila., 1926.
Penfield, W. Nelson's Loose-Leaf Surgery, N. Y., 1927.

‡ Cajal, S. Ramon y. *Trab. d. Lab. d. Invest. Biolog. d. l. Univ. d. Madrid*, 1913, ii, 255.

somes, and have more irregular expansions. They normally have no fibers but develop them under numerous pathological conditions.

Fibrous astrocytes (Fig. 2) contain long slender fibers of Ranvier-

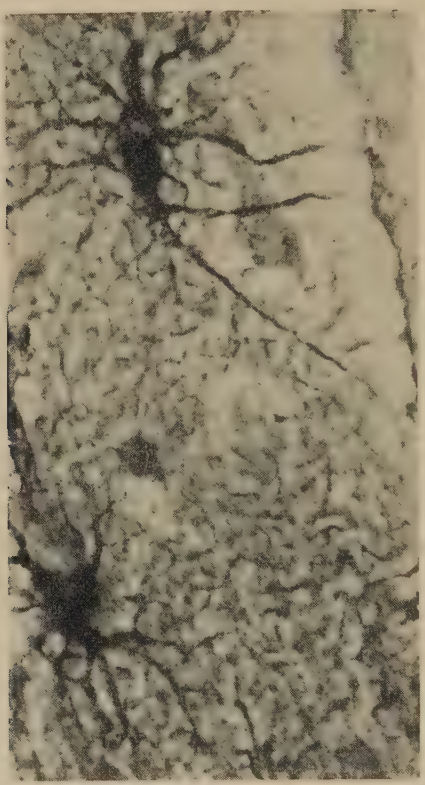

FIG. 1. Protoplasmic astrocytes.

Weigert which pass through the cell body and out into the expansions. Under normal circumstances the fibers are not divorced from the cell and gliosomes can be seen to accompany them. Selective fiber stains such as Weigert's may give an erroneous appearance of fiber divorcement.

Pathological changes occur rapidly in astrocytes, particularly the ameboid change of Alzheimer (clasmatodendrosis of Cajal), (Fig. 3). This may appear as an acute change in various toxic conditions of the nervous system, as an agonal change and also as a post-mortem change. It is found earlier in the white matter and often it affects one cell and spares the next. In staining human material the characteristics of this change must be kept constantly in mind. The fragmented expansions (Fullkörperchen) seen in Figure 3 may otherwise be taken for artifacts.

2. Oligodendroglia (oligoglia, Robertson's mesoglia). These cells were first described by Robertson in 1900.* He used a platinum method which proved so unreliable as not to warrant description in these pages. Del Rio-

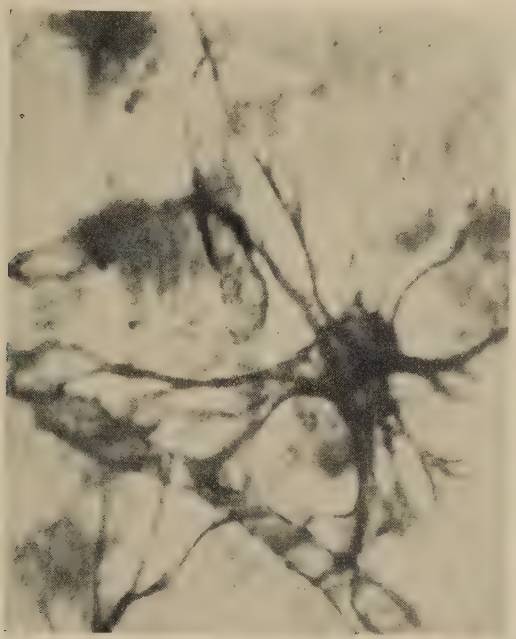

FIG. 2. Fibrous astrocyte.

Hortega in 1921 provided a better method and a full description of these cells.

Oligodendroglia cells are by far the most numerous of the supporting cells in the nervous system. They occur in rows in the white matter (Fig. 4) where their expansions interlace about the medullary tubes. They thus hold a position relative to nerve-fibers, comparable to that of the sheath of Schwann cells in the peripheral nervous system. They are also present about nerve-cells (Fig. 5) appearing as "satellites" especially in the neighborhood of the axon hillock. These cells contain gliosomes similar to astrocytes. They do not possess perivascular attachments and form no fibers. They are much smaller than most astrocytes. Their small rounded nuclei are seen in ordinary stains without cytoplasm.

Normal oligodendroglia are not ordinarily seen in human material unless death is sudden and the autopsy early. Material obtained at operation and following accidental death contains oligodendroglia similar to

* Robertson, W. *Scot. Med. & Surg. J.*, January, 1899.
Robertson, W. *J. Med. Sc.*, October, 1900, p. 724.

that of sacrificed animals. If death is preceded by coma or deep stupor there will be found acute swelling of oligodendroglia (preameboid glia),* (see Figs. 6 and 7). If there is a toxic or infectious cerebral condition this swelling

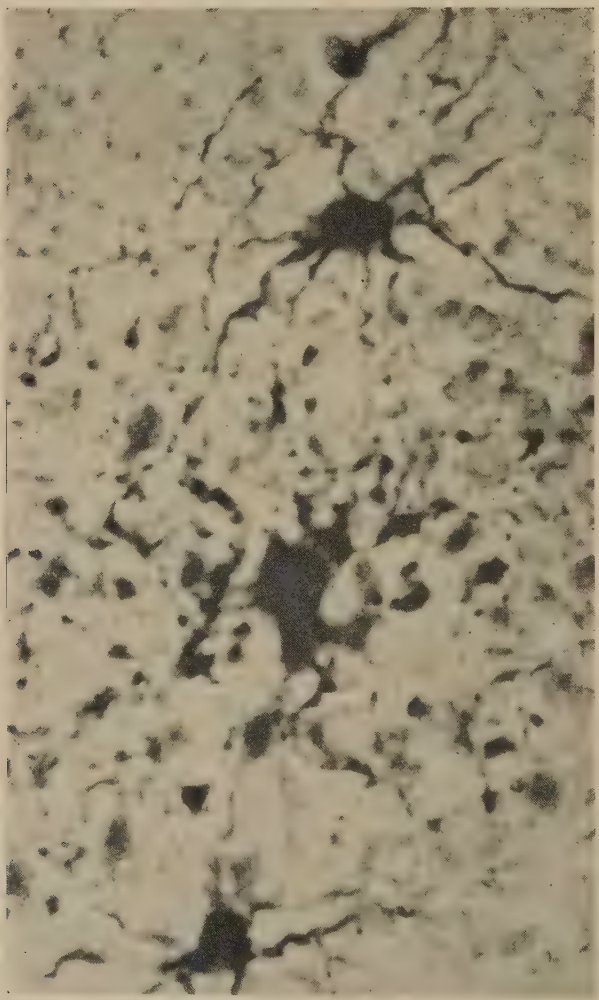

FIG. 3. Clasmatodendrosis of fibrous astrocyte. Also unaltered cell.

is extreme and may go on to cell destruction before death. This acute swelling occurs also as an autolytic process. It is a more sensitive change than clasmatodendrosis and occurs earlier but as the result of the same influences. In certain conditions also there is an increase in the number of perineuronal oligodendroglia amounting to a "satellitosis."

* Penfield, W. and Cone, W. *Arch. Neurol. & Psychiat.*, 1926, xvi, 131.

3. **Microglia** (mesoglia, Hortega cells). These cells were first described under normal conditions by Del Rio-Hortega in 1919.* In their pathological forms they have long been recognized as rod cells and compound granular

FIG. 4. Oligodendroglia of the white matter (interfascicular).

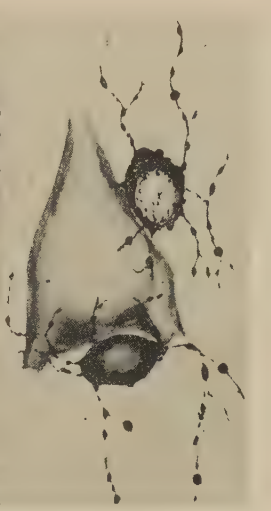

FIG. 5. Perineuronal oligodendroglia (satellite).

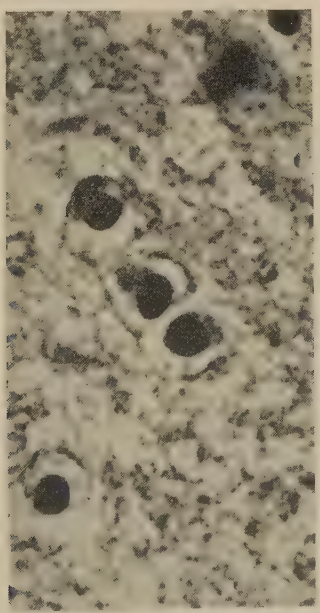

FIG. 6. Acute swelling of intrafascicular oligodendroglia.

* Del Rio-Hortega, P. *Boletin d. l. Soc. Esp. d. Biolog.*, 1919, ix, 68.

corpuscles. These cells are small in comparison with astrocytes. They have irregular protoplasmic expansions (Fig. 8) with stubby, spinelike projections. The nucleus is usually elongated or triangular and contains a heavier

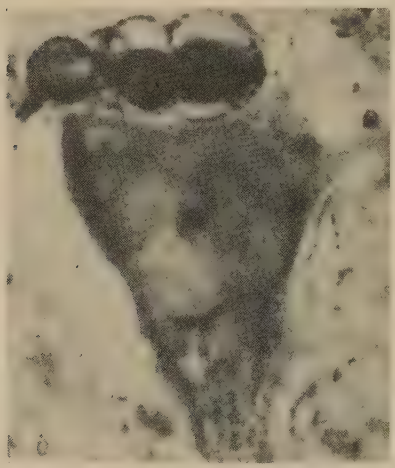

FIG. 7. Acute swelling of perineuronal oligodendroglia.

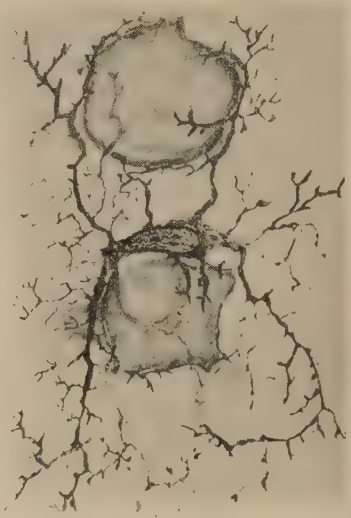

FIG. 8. Microglia.

chromatin net than that found in neuroglia. There are no gliosomes and no fibers. The cells are not sensitive to the same toxic, agonal and autolytic influences as neuroglia.

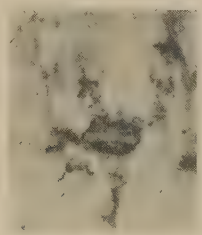

FIG. 9. Intermediate form of microglia near brain wound.

FIG. 10. Compound granular corpuscle.

The development of these cells is quite different from that of neuroglia, both forms of which develop from ectodermal spongioblasts. Microglia seems to develop from the pia mater and possibly vascular adventitia and is therefore probably of mesodermal origin. These cells correspond to macrophages elsewhere in the body and may be grouped in the reticulo-

endothelial system. The methods given here for microglia will stain the other elements of that so-called system. Microglia cells react promptly to destructive processes* passing rapidly through transitional forms (Fig. 9) to become phagocytic "Gitterzellen" (Fig. 10).

The methods to be described may be divided into three groups according to the cells stained: (a) astrocytes, (b) oligodendroglia, (c) microglia. By the first group all types of astrocytes may be stained but small modifications of these methods may demonstrate the protoplasmic type better than the fibrous, and vice versa. In general, the pathological forms of all three types of cells are more easily demonstrated by the metallic methods than are the normal forms.

Oligodendroglia may be impregnated in the gray and the white matter with equal facility. Microglia cells are studied more easily in the gray matter where they are much more numerous. When oligodendroglia is well stained microglia is usually stained also. But either cell group may be stained with exclusive selectivity. At times when using silver carbonate, astrocytes may be incompletely stained along with the two groups of smaller cells.

II. An Outline for the Routine Study of the Interstitial Cells

It is necessary in most pathological laboratories to have a routine procedure for use in the study of the brain and spinal cord. When certain aspects are of importance, as in a particular specialized research problem, the material may be treated with a view to the exclusive demonstration of one cell group as described in the methods below. As a rule, however, it is necessary to compromise to some extent so as to use various methods with the best average result. The following outline may be found useful for that purpose:

Outline. Remove the brain and cord as soon after death as possible. It is preferable to inject the carotid artery early with 10 per cent formol to secure the best possible cell preservation. If this is not feasible excellent and immediate fixation of the whole brain may be accomplished by injecting the basilar artery of the brain after the cut branches of the circle of Willis have been ligated. This injection does not seem to prevent good results even with Nissl's or Cajal's methods if blocks are taken for these procedures immediately after the removal of the brain, and the preservation of neuroglia is thereby greatly improved.

Remove some blocks from the brain at once and place them in alcohol for Nissl's stains. (For a thorough survey of the interstitial cells of the various areas Orton's method† of taking blocks is orderly and very useful.) Place other blocks in formalin-ammonium-bromide solution in the 38°c. incubator for Cajal's gold chloride method (page 386). These blocks

* Penfield, W. *Am. J. Path.*, 1925, i, 77.

† Orton, S. T. *Am. J. Insan.*, 1912, lxix, 429.

are cut and stained at the end of forty-eight hours and if desired some of the sections may be stained for oligodendroglia by Hortega's method (1) at the same time. Place the brain and cord in 10 per cent formalin. The removal of these preliminary blocks may be omitted if it is desired to keep the brain intact until it can be photographed. But results for these two methods are then not so good.

On the fifth day make gross photographs and cut the brain. Blocks are then taken for any of the various methods as outlined in Chapter 1. Blocks are also cut on the freezing microtome and the sections stained by each of the following methods: Globus modification of Cajal's gold chloride method (page 369); Penfield's second modification of Hortega's silver carbonate method for both microglia and oligodendroglia (page 379); Bielschowsky's or preferably Hortega's method for neurofibrils and Hortega's or Perdrau's method for connective tissue. If the gold chloride method for astrocytes should have proved unsatisfactory the method of Hortega (page 374) can be tried on the sections from the same block or on other blocks after longer fixation.

B. NEUROGLIA ASTROCYTES (MACROGLIA)

The bichromate silver method of Golgi* was long the best staining procedure for classical neuroglia and because of the appearance of these cells they were considered as independent structural units. The splendid method of Weigert then came into use and showed neuroglia fibrils with such selectivity that they came to be considered as extra-cellular structures.

There followed many other methods, some of which added important details, while others stained neuroglia so indistinctly that these cells seemed to merge in with a vague diffuse syncytial network. There are many important methods which cannot be described here, such as Weigert's,† Benda's,‡ Alzheimer's,§ Mallory's phosphotungstic hematoxylin method (p. 308), Anglade and Morel's Victoria blue method, Lhermitte's,‖ Bailey's,¶ et al.

The method of Achúcarro provided in 1911 a very valuable means of studying astrocytes. But the advent of Cajal's gold chloride method in 1913 demonstrated these cells both protoplasmic and fibrous with

* Golgi, C. Opera Omnia. 3 vols. Milan, 1903.

Lee, A. B. The Microtomist's Vade-mecum, Ed. 9, London, 1928.

† Weigert, C. Beitrage zur Kenntniss der normalen menschlichen Neuroglia. Frankfurt, 1895.

‡ Benda. See chapter on "Neurogliafarbung." Ebzykl. mik. Technik, 1910, ii.

§ Alzheimer, A. Histolog. u. histopath. Arb. Nissl-Alzheimer, 1910, iii, 401.

Spielmeyer, W. Technik der microskopischen untersuchung des Nervensystems. Berlin, 1914.

‖ Roussy, C. and Lhermitte, J. Les techniques anatomopathologiques du Systems nerveux. Paris, 1914.

¶ Bailey, P. *J. Med. Research*, 1923, xliv, 73.

unequaled clarity and selectivity. The cells could be seen as cell entities stained completely with gliosomes, glia fibrils, perivascular feet, etc. The method of Del Rio-Hortega provided in 1917 another photographic procedure which when successful cannot be excelled and which is to be preferred to the gold method if the tissue has been long in formalin.

I. Cajal's Gold Chloride Sublimate Method for Neuroglia Astrocytes* (G. C. S.)

1. The Method.

(1) *Harden.* Blocks of tissue not over 5 mm. in thickness should be removed from the central nervous system as soon as possible after death and placed in formalin-ammonium-bromide solution (solution c under "Solutions" p. 386). Allow blocks to harden in this solution at room temperature for four to twenty-five days. In general, protoplasmic astrocytes stain better with a short fixation time and fibrous astrocytes after longer fixation.

(2) *Cut.* Sections are cut on the freezing microtome at about 25 μ and placed directly in distilled water to which a few drops of formalin have been added to prevent swelling of sections.

(3) *Wash.* Pass the sections rapidly through two changes of distilled water in Petri dishes and place them in the gold bath.

(4) *Stain.* Place sections in a flat dish which is filled to the depth of about 1 cm. with gold chloride sublimate solution (solution d). Flatten out each section on the bottom of the dish so that it is not folded or overlapped by other sections. Place the dish in the dark, preferably in an oven at a temperature of 22°c., for human cerebrum where four to six hours will be required (see technical discussion). When sections begin to turn an intense purple remove them.

(5) *Fix.* Transfer sections from gold bath to Cajal's fixing bath (solution e 386). Leave them here six to ten minutes.

(6) *Wash.* Wash sections in 50 per cent alcohol and mount on glass slip.

(7) *Dehydrate.* Blot section on slip and dehydrate with absolute alcohol.

(8) *Clear.* Oil of origanum and xylol.

(9) *Mount.* Canada balsam.

2. Discussion of the Steps of the Method. (1) *Hardening.* Ammonium iodide may be substituted for the bromide, this combination being better than formalin alone. Also, according to Cajal 25 per cent of methyl alcohol may be used in the fixative, or 2 per cent of acetanide, also carbamide nitrate in the same strength. This last substance prevents granular appearance but lessens the energy of the impregnation. If it is desired to study fibers of Ranvier-Weigert, this last salt may be used to good advantage.

* Cajal's successive descriptions of this method† provide advice and warnings concerning the method but make no fundamental alterations in original procedure. If the results are not uniformly good, reference may be made to the original descriptions by this master of technique, but as much as possible of his advice is included here. Where there is a difference the conclusion last published is given.

† Cajal, S. Ramon y. *Trab. d. Lab. d. Invest. Biolog. d. Univ. d. Madrid,* 1913, ii, 219.
——— *Ibid.,* 1916, xiv, 155.
——— *Ibid.,* 1920, xviii, 129.

In case the tissue has been hardened in formalin without ammonium bromide, successful staining may at times be obtained; indeed, this was the fixative first used by Cajal. We have occasionally succeeded by placing old formalin sections in formalin-ammonium-bromide for a time and proceeding as usual.

The optimum duration of hardening for the nervous system of newborn mammals was found by Castro* to be three to four days in formalin-ammonium-bromide. For the human olfactory lobe† he secured good results by substituting urea nitrate for ammonium bromide in hardening as follows: formol, 15 c.c.; water, 100 c.c.; urea nitrate, 1 to 2 gm. He also successfully employed for this tissue: formol, 15 c.c.; water, 100 c.c.; commercial ammonium carbonate, 2 gm.

(2) *Sections.* In the opinion of Cajal the most favorable results are obtained with thick sections which also permit one to follow the sprawling astrocyte expansions through their full extent. In our hands, sections cut at about 15μ are preferable for staining and make photography easier.

Cajal has pointed out that one weakness in the method is that the superficial tissue is apt to stain less well than the deeper tissue, due to the fact that the more energetic action of formalin at the surface carries the superficial tissue too far, destroying the aurophilic properties of the astrocytes by the time the deeper cells are in a condition suitable for staining. This may be avoided by leaving the pia intact or covering with a layer of blood or other substance.

(3) *Staining.* The importance of the temperature of the gold sublimate bath must not be overlooked. Cajal concluded that 18 to 22°C. is the proper temperature for the human cerebrum. At such temperatures the time consumed is usually four to eight hours. For mammals low in the phylogenetic scale and for birds, reptiles and fish the optimum temperature is 25 to 35°C. For cerebellum, bulb and spinal cord, a somewhat higher temperature of gold bath is desirable according to Cajal, i. e., 25 to 28°C. In the pineal gland, Del Rio-Hortega was able to stain neuroglia only if a temperature of 27 to 30°C. was used. Castro prefers for the olfactory bulb of cats, dogs and rabbits the temperature of 24 to 27°C. and for the human olfactory lobe 35 to 40°C. which accomplishes the reaction in less than an hour. For the nervous system of newborn mammals he advises 24 to 26°C.

Although Cajal advises placing the gold bath in the dark, we have not observed any detriment to the process when it is accomplished in daylight. When the sections are becoming purple it is well to remove and mount a single section to see if the reaction is complete. The astrocytes should stand out like black stars even when examined wet under the microscope.

If the room is cold the sublimate may be doubled, or the gold doubled and the sublimate tripled. Brevity and energy of reaction may also be obtained by adding 2 to 3 drops of a 1 to 1000 solution of erythrosin.

* Castro, F. de. *Trab. d. Lab. d. Invest. Biolog. d. l. Univ. d. Madrid,* 1916, xiv, 83.

† *Ibid,* 1920, xviii, i.

(4) *Fixing.* As first used by Cajal the sections were not fixed but he found the preservation not perfect without fixation. It is our custom to wash before passing the sections into the fixative as is done by Del Rio-Hortega. We have also substituted ordinary photographic hyposulphite of soda, 5 per cent, for the more complicated fixing bath of Cajal with better results in our hands.

(5) *Dehydration and Mounting.* It is our custom to dehydrate clear and mount after the fashion of Del Rio-Hortega in his silver methods, using carbol-xylol-creosote to clear after 95 per cent alcohol followed by balsam. Some of our preparations thus mounted are perfectly preserved for over three years.

a. Modification of Globus. Globus* has provided a method of rendering tissue hardened in formalin available for staining with gold, which to quote from his description is as follows:

(1) Prepare frozen sections of formaldehyde-fixed material at a thickness of from 15 to 30μ.

(2) Wash quickly in several changes of distilled water.

(3) Place in a 10 per cent solution of strong ammonia water for twenty-four hours at room temperature, or for shorter periods in an incubator.

(4) Carry rapidly through two changes of distilled water.

(5) Place in a 10 per cent solution of (pure, 41 per cent) hydrobromic acid and let it remain there from two to four hours.

(6) Wash quickly in two changes of distilled water, to which a few drops of ammonia water are added and place section in gold chloride solution as usual.

In our experience, this works out very well provided the tissue has not been too long in formalin.

3. Underlying Principles of the Gold Sublimate Method. The chemical formulas involved in the reaction are unknown. As has been said, results which are at times quite favorable may be secured after fixation in formalin alone. The addition to the fixative of ammonium bromide, ammonium iodide or potassium iodide improves the vigor of the eventual impregnation and lessens the tendency to granular staining.

The presence of the corrosive sublimate in the gold bath seems to be indispensable to a successful outcome. Gold chloride alone will color the tissue diffusely. The addition of mercuric chloride seems to render the astrocytes aurophilic and to accelerate the deposit in these cells of metallic gold. It is possible that mercury, which is capable of going into solution with the gold, withdraws the latter metal from all the cells except astrocytes. The action of the final fixing bath is only to preserve the preparation.

In the protoplasmic astrocytes, as pointed out by Cajal, the staining affinity of the terminal expansions seems to be a little different from that of the cytoplasm of the cell body, for when these expansions are energetically impregnated the cell body and nucleus are more faintly stained, and vice versa.

* Globus, C. *Arch. Neurol. & Psychiat.*, 1927, xviii, 263.

This is not true of the fibrous astrocytes but there is definite difference in reaction between the fibrous and the protoplasmic astrocytes. In general the latter cells in the gray matter reach their optimum for staining after two or three days of hardening, while the fibrous astrocytes of the white matter stain best at a later period and continue to be aurophilic after the protoplasmic cells have become refractory to gold.

The action of the formalin, if allowed to continue, eventually renders all the astrocytes refractory to gold.

4. Results. With an insignificant amount of labor the astrocytes (classical neuroglia) are stained in an exquisitely selective manner. The astrocytes of the white matter are shown completely with their fibers and particularly their perivascular feet. If the staining is not intense the fibers can be seen to pass through the cytoplasm of the cell body and out into the expansions. With more diffuse impregnation the individual fibers are lost sight of and only the shape and length of the expansion indicate that it is fibrous. It must be remembered that astrocytes often undergo an agonal or post-mortem change (clasmatodendrosis) consisting of swelling and fragmentation of the expansions.* Autopsy material must therefore be obtained as quickly as possible after death.

But it is in the staining of the protoplasmic astrocytes that the gold-sublimate method excels all previous methods. The protoplasm can be followed out to terminal expansions and the perivascular feet are easily demonstrated. Gliosomes, small, smooth, oval or round granules, may be at times stained with great distinctness.

Neuron bodies may be made out very indistinctly but axons and myelin sheaths are completely unstained. Oligodendroglia cells are unstained, although a halo is seen about these nuclei. Likewise microglia (mesoglia of Hortega) are unstained except for their elongated nuclei. The lepto- and pachymeninges are invisible, as are also the blood vessels. Capillaries may be outlined by well-stained perivascular glia feet.

The method works particularly well on human material. Of laboratory animals, dogs and cats give particularly clear pictures. Rabbits in our experience give only fair results. Del Rio-Hortega's method is preferable for these last animals. Splendid impregnations will be secured with newborn animals, astroblasts being well demonstrated. When the method is used on gliomas only those cells are stained which have progressed to the astrocyte stage. The more embryonic forms do not stain at all. It is useful therefore as an indicator of the degree of differentiation of these neoplasms. The giant cells in spongioblastoma multiforme may stain and the cells of the astrocytomas should stain well, although in our hands, so far, these tumors are sometimes refractory to gold, while the phosphotungstic acid method of Mallory may succeed better. The astrocytes that appear in

* Penfield, W. and Cone, W. *J. f. Pyschol. u. Neurol.*, 1926, xxxiv, 204.

ependymomas stain well, while none of the cells of medullo-blastomas are impregnated.

The procedure can be carried out in a routine standard manner if the proper chemicals are obtained, if the glassware is scrupulously clean and if attention is paid to the above advice with regard to time of fixation.

One difficulty with the method is that occasionally the astrocytes, though selectively stained, are quite granular. This may be due, among other causes, to leaving sections too long in the gold bath, too long hardening, or overheating.

The best results will be obtained with the use of formalin-ammonium-bromide as fixative. Good results may be obtained, however, with the use of the Globus modification after formalin fixation, provided the time of formalinization is not too great. In our experience, with an occasional exception, material which has hardened in formalin longer than six weeks gives only fair results with gold chloride, whatever modification be employed.

5. Routine Use of the Method. The routine procedure which we have gradually come to use during the past four years will be outlined below. We do not propose it as a new modification. In addition to the personal alterations there are some details in it derived from Del Rio-Hortega and there is an important step borrowed from Globus.

(1) *Harden* thin blocks in formalin-ammonium-bromide solution one day in the incubator at 38°c.

(2) *Cut* sections on the freezing microtome at 15 μ thickness and place directly in 1 per cent formalin.

or

(1) *Harden* the whole brain in 10 per cent formalin for a week at room temperature, preceded, if possible, by injection of formalin at the earliest possible moment after death. In experimental animals the injection is made while the animal is under ether anesthesia. If a pressure of about 2½ meters is used it is not necessary to wash out the vessels with any other solution. When the brain has been thus injected the second step should be taken early, even before twenty-four hours.

(2) *Cut* as above, wash and follow the Globus procedure by placing sections in a closely covered vessel containing distilled water to which is added about 1 drop of strong ammonium hydroxide for every cubic centimeter of water. Leave in this overnight. Then wash sections quickly and place them in 10 per cent hydrobromic acid for one hour in the incubator at 38°c.

(3) *Wash* rapidly in two changes of distilled water.

(4) *Impregnate.* Place not over six sections in a flat porcelain dish provided with porcelain cover and containing about 25 c.c. of freshly prepared gold sublimate solution. This dish is used for no other purpose and the glassware for preparing gold bath is used for nothing else as a further security against contamination.

The bath is prepared as described under Solution d, except that the amount of mercury bichloride is doubled, i. e., mercury bichloride 1 gm.; water 50 c.c.; and gold chloride (1 per cent) 10 c.c.

The sections are laid flat on the bottom of the vessel and kept in the dark. When the purple begins to appear, one section is removed and examined wet and if the astrocytes

are seen, it is mounted. The optimum color is usually a reddish purple. A little reddish deposit in the solution is of no consequence, but if a scum begins to form the sections should be removed.

The bath is at room temperature but during the heat of a New York summer the results are apt to be unsatisfactory.

(5) *Wash* in distilled water.

(6) *Fix* the sections in 5 per cent sodium hyposulphite (photographic "hypo").

(7) *Wash* thoroughly in several changes of water. Extra sections may be placed in 1 per cent formalin and preserved there for long periods.

(8) *Dehydrate* in 95 per cent alcohol after floating the sections from water on to the slip.

(9) *Clear* with Hortega's carbol-xylol-creosote mixture (Solution f). Blot as soon as the section is clear and flattened.

(10) *Mount* in Canada balsam.

II. General Observations on Silver Staining*

In general, each of the silver methods should be carried out with a standardized technique. There are minor differences between them and it may be necessary to try small variations at times to achieve success, but certain rules must be constantly observed. In all the variations of the silver carbonate method, as well as the tannin silver method, the equipment of the table is much the same.

It is best to carry out the silver procedures at the worker's own desk where a microscope can be used to determine the optimum impregnation times, and all solutions are within reach, as in Figure 11. The desk and shelves in this illustration are planned especially for silver staining.

In many of the procedures the sections can be cut, stained, mounted and studied in the same working period. It is only thus that the best results are obtained, for defects in the desired staining result often can be remedied by small variations in the procedure instituted at once.

A bottle of doubly distilled water should be on the table. It stands in the waste jar until it is called into use. The following drop bottles are needed and may conveniently be kept in sockets in a block of wood that may be moved about at will: alcohol 96 per cent; carbol-xylol-creosote; xylol; ammonium hydroxide; pyridine.

For washing sections, Petri dishes which contain 70 c.c. are used. The small glass dishes used for silver, for toning gold and for "hypo" solutions contain 15 c.c.

When it is desired to heat the sections they are placed in a glass of solution upon a tripod and asbestos sheet (Fig. 12) over an alcohol lamp. The glass dish should be filled and covered with a watch glass so that a bubble of air remains inside capable of stirring up the sections when the whole is shaken. The heat should never exceed 50°C., a temperature at which the glass begins to feel disagreeably hot against the finger.

A powerful artificial light should be used with a blue filter, as many of the sections are quite thick. For examining wet sections a microscopic magnification of about 150 is satisfactory.

The silver of Bielschowsky,§ though designed for staining neurofibrils, has been used by means of numerous modifications for the staining

* Metallic methods have been well reviewed by Carleton† and by Da Fano.‡

† Carleton, H. Histological Technique. Lond., 1926.

‡ Lee, A. The Microtomist's Vade-mecum. London, 1928.

§ Bielschowsky, M. *J. f. Psychol. u. Neurol.*, 1904, iii, 169.

of neuroglia. Most successful among these is perhaps the method of Achúcarro. This silver solution is prepared by precipitating the silver from silver nitrate solution with sodium hydroxide and redissolving the precipi-

FIG. 11. Desk and cupboard for silver staining.

tate with ammonia. The "silver carbonate solution" of Del Rio-Hortega is obtained by precipitating the silver from the silver nitrate solution with sodium carbonate and redissolving that precipitate with ammonia. In

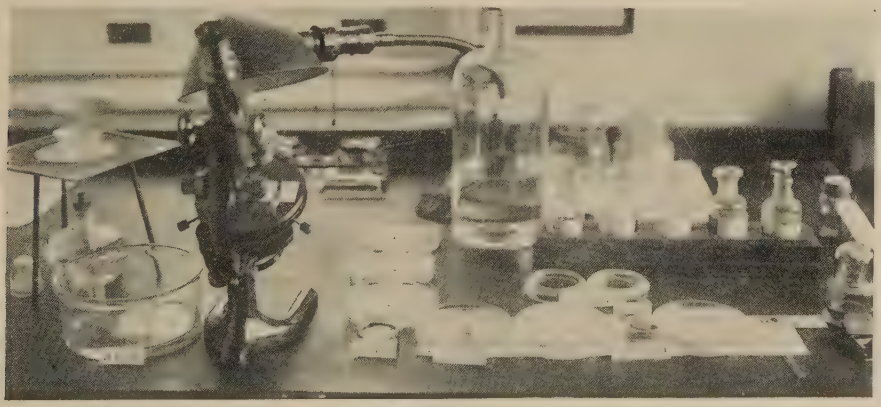

FIG. 12. Same desk, one of Hortega's silver carbonate methods in process.

the Achúcarro method the sections are passed through a mixture of tannic acid and ammonium hydroxide to prepare them for the silver bath. After the bath the silver in the tissues is reduced in formaldehyde.

In the Hortega methods the sections are prepared in a variety of ways according to which type of cell is to be demonstrated. After passing through the silver bath they, too, are plunged into formaldehyde for reduction.

For these silver procedures ammonium bromide, used to prepare the tissue, improves the staining of astrocytes, just as it does in Cajal's gold chloride method. But it is not indispensable for any of them. In the staining of oligodendroglia and microglia by ammoniacal silver carbonate this preliminary use of ammonium bromide in some form seems to be indispensable.

Our knowledge is not sufficiently advanced to permit chemical analysis of these metallic methods. As Liesegang* concluded, after studying silver staining, a new gel chemistry is required. Formalin in the tissue seems to act as a mordant for the silver. The various accessory mordants used in different modifications serve to render some particular cell group selectively argentophilic, so that when the sections are passed to the reducing formalin silver stains that cell group takes up only the silver. The section after being mordanted and passed through silver has been likened by Liesegang to a photographic plate which had been exposed but not developed. Reduction of the section and development of the plate demonstrate the picture.

III. Del Rio-Hortega's Silver Carbonate Method for Astrocytes,† (S. C. A.) (Fig. 13)

1. Method.

Harden. The tissue should be cut in blocks less than 1 cm. in thickness and hardened in formalin-ammonium-bromide solution (c). Duration in this fixative should be about twenty to forty days at room temperature for best results with the protoplasmic astrocytes of the gray matter and something over a month's duration for good results with fibrous astrocytes. It is difficult to give definite dates for hardening as the optimum duration is not constant. The time of hardening may be shortened by placing the tissue in an incubator at 38°c. Good results in both types of astrocytes may then be obtained at the end of two to three weeks.

Results, sometimes quite good results, may be had after hardening in 10 per cent formalin even for very long periods. Staining of protoplasmic astrocytes may be obtained as early as the third day after simple formalin fixation, and the modification of Globus may be used here to bromurate the formalin fixed tissue (p. 369). Also it is sometimes very helpful to place formalin-fixed sections in Cajal's reinforcer solution i for four hours at 38°c. before beginning the staining.

Section. Cut sections on the freezing microtome at 15 to 25 μ and receive in water containing a few drops of ammonia.

(1)‡ *Wash.* Wash well in four changes of water (Figs. 12–1, 2, 3) to get rid of all formol.

(2) *Stain.* Place six to twelve sections in ammoniacal silver carbonate (lithium) (solution g) in a small glass dish which contains about 10 c.c. Add a few drops of pyridine

* Liesegang, R. *Kolloidchem. Beihefte,* 1912, iii, 1.

† Del Rio-Hortega, P. *Trab. d. Lab. d. Invest. Biol. d. l. Univ. d. Madrid,* 1917, xv, 367.

‡ A diagrammatic representation of this method is seen in Figure 13. Numbers in the diagram correspond with those of the description.

to prevent the formation of a scum on the surface. Cover with a watch glass leaving a bubble and place over the alcohol flame (Fig. 12). Heat to 45 or 50°C. shaking from time to time. Leave sections here, usually three to five minutes, until they become dark amber

FIG. 13. Del Rio-Hortega's silver carbonate astrocyte stain. 1. Water. 2. Silver carbonate (lithium), pyridine, alcohol. 3. Water. 4. 1 per cent formol. 5. Water. 6. Gold chloride. 7. "Hypo." 8. Water.

in color and the liquid takes on a grayish color. (The liquid may become brownish in case the sections have been insufficiently washed.)

If fixation has been short or the first result is unsuccessful remove the sections and heat in a second dish of silver solution similarly prepared except that 12 drops of 96 per cent alcohol are added. Alcohol if desired may be added also to the first silver. Remove sections when an amber color is secured.

It is well to carry one section through to reduction and float onto a slip for microscopical examination to see whether the impregnation is complete or not and whether a second heating in silver is necessary.

(3) *Wash.* Place sections in distilled water. Allow them to fall to the bottom. Then raise them with the glass rod, wash quickly and plunge them into the reducer. Too long washing gives a pallid result.

(4) *Reduce.* One per cent formalin (10 per cent may be used, but is no better in our experience) is used as "reducer." The reduction takes place at once.

(5) *Wash.* Wash well.

(6) *Tone.* Place sections in gold chloride solution (h). Leave them here a few minutes till they become gray, then heat, not exceeding 50°C. until the sections become a dark purple.

(7) *Fix.* Pass sections directly to 5 per cent hyposulphite of soda. Leave them here until flexible, about one-half a minute.

(8) *Wash* in water, mount, dehydrate, clear, etc. as for the other methods

2. Results. The general background should be unstained and the astrocytes clearly outlined, the nucleus and pigment granules being clearly stained, and the fibers in the fibrous astrocytes may be distinguished as they pass through the cytoplasm of the cell body. Gliosomes are rarely stained.

When perfectly successful the result is brilliant, the astrocytes are stained completely and the contrasts such that photography is quite easy. On the other hand, the method is variable and less reliable than that of Cajal's gold sublimate method. It is the more reliable of the two methods when the nervous tissue has been long fixed in formol.

For pathological astrocytes, where there has been hypertrophic gliosis, the silver carbonate method gives particularly good results, often staining only the enlarged cells but staining them with the greatest clarity even after ordinary formalin fixation.

If it is desired to study the astrocyte cytoplasm it is better to omit toning the sections and to mount after reduction and washing in water.

On the other hand, toning reinforces the glia fibrils and gives better preparations for routine work. If formalin is used for hardening without ammonium bromide the neurofibrils are likely to be stained rather too much.

3. Modification of Cajal. *Ammoniacal Silver Oxide Method for Astrocytes.** This is in principle a modification of the foregoing silver carbonate method of Del Rio-Hortega. In our hands it has proved inferior to the silver carbonate method or the gold chloride method. Cajal recommended it for the study of general paresis, as it seems to stain pathological glia well, just as does Hortega's method.

(1) *Harden.* Tissue as fresh as possible placed in formalin-ammonium-bromide for two to twenty-five days.

(2) *Section.* Frozen sections at 25 to 35 μ are placed in formalin-ammonium-bromide.

(3) *Reinforce.* For more energetic staining (particularly of the cell body) the sections should remain four hours at room temperature (or preferably in the incubator) in the reinforcer, solution i. To stain glia fibrils leave in reinforcer a short time only.

(4) *Wash.* Wash rapidly in three changes of distilled water.

(5) *Stain.* Place in the following ammoniacal silver bath:

Ammoniacal silver	10 c.c.
Water	10 to 12 c.c.
Pyridine	7 to 10 drops

Note. For more energetic staining the silver bath may be used without dilution. Alkalinity or precipitation weaken it.

(6) *Wash.* Wash rapidly in two changes of distilled water.

(7) *Reduce.* Plunge sections in 5 per cent neutral formalin.

(8) *Tone.* Twenty minutes at room temperature.

(9) *Fix.* In hyposulphite of soda.

(10) *Wash.*

(11) *Dehydrate, clear and mount.*

IV. Achúcarro's Tannin-silver Method†

This method, published in 1911, precedes those of Cajal and Del Rio-Hortega. It gave good results at times but was rather unreliable. Certain modifications were used in practice by Achúcarro particularly for protoplasmic astrocytes and these were published by his pupil Del Rio-Hortega‡ briefly as follows:

1. The Method.

(1) *Harden.* Pieces not over 2 to 3 mm. in thickness are placed in 20 per cent formol to which enough ammonia has been added to give an alkaline reaction with litmus paper.

(2) *Section.* Frozen sections not over 10 μ in thickness.

(3) *Mordant.* The sections, well flattened, are heated in 10 per cent solution of pure tannic acid for ten minutes, preventing bubbles.

* Cajal, S. Ramon y. *Trav. d. Lab. d. Recb. Biol. d. l. Univ. d. Madrid*, 1925, xxiii, 157.

† Achúcarro, N. *Bol. d. l. Soc. Esp. d. Biolog.*, 1911, i, 139.

‡ Del Rio-Hortega, P. *Trab. d. Lab. d. Invest. Biol. d. Univ. d. Madrid*, 1916, xiv, 181.

(4) *Wash.* After mordant has cooled wash the sections in water containing a few drops of ammonia until they become flexible.

(5) *Impregnate.* Place sections in bath made by adding 3 to 4 drops of Bielschowsky's

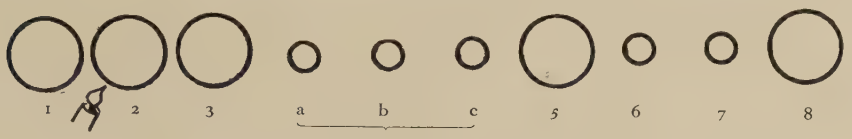

FIG. 14. Del Rio-Hortega's first variant of Achúcarro's method. 1. Water. 2. Tannic acid. 3. Water, ammonia. 4a, b, c. Silver oxide, ammonia. 5. Water. 6. Gold. 7. "Hypo." 8. Water.

ammoniacal silver (solution k) to 10 c.c. distilled water. Remove the sections when the white matter begins to take on color.

(6) *Reduce.* Place in formalin prepared as for the hardening, for five minutes. This method demonstrates in general with great distinctness protoplasmic constituents such as granulations, centrosomes, myofibrils, glia fibrils and mitochondria, in addition to neuroglia and myelin sheaths. The *first* variant of Hortega described below, however, demonstrates the same structures more consistently.

2. Modification of Del Rio-Hortega. *The "First Variant" Method* (Fig. 14).

Harden at least ten days in 10 per cent formalin.

Section. Frozen sections.

(1) *Wash.* Water.

(2) *Mordant* the sections (cut on the freezing microtome) in 3 per cent tannic acid solution at 50°C. for five minutes.

(3) *Wash* them in 20 c.c. distilled water containing 4 drops of ammonia until their flexibility and elasticity return.

(4) *Impregnate* by passing the sections in groups of 3 or 4 through three dishes A, B and C, arranged in series and each containing 10 c.c. distilled water and 1 c.c. ammoniacal silver (12 to 15 drops), (k). Leave the sections in first silver until the solution takes on a dark color, then pass them into the next. In the last solution the sections should be a deep yellow color while the solution itself is nearly colorless. A fourth dish of solution may be used if necessary. (5, 6, 7, 8. Numbers refer to those used in Fig. 14.) Wash, tone in gold and fix in hyposulphite; wash. This is a good cytological stain for various purposes and may be used particularly for fibrous astrocytes.

The second and third variants of this method were devised by Del Rio-Hortega for staining collagenous fibers of connective tissue. However, the third variant also stains fibrous neuroglia but will not be detailed here. The fourth variant described by him at a later time is a useful method for protoplasmic astrocytes.

3. Modification of Del Rio-Hortega.* *The "Fourth Variant" Method.*

Harden in formalin for a long or short period. Cut sections as usual and place them for a few minutes at 45 to 50°C. in tannin-ammonium-bromide mordant (solution I). Wash in ammonia water until the sections are flexible and transparent. Impregnate in ammoni-

* In a personal communication Percival Bailey states that he has found this method very useful to demonstrate cytoplasmic outlines of cells in brain tumors.

acal silver solution diluted as above until they are yellowish. Wash for a few seconds in water and reduce in 20 per cent formalin which has been neutralized by the addition of chalk some days before. Wash, tone and fix as above.

FIG. 15. Del Rio-Hortega's silver carbonate method for oligodendroglia. 1. Water. 2. Silver carbonate (strong). 3. Water. 4. 1 per cent formol. 5. Water. 6. Gold. 7. "Hypo." 8. Water.

C. OLIGODENDROGLIA

V. Del Rio-Hortega's Silver Carbonate Method for Oligodendroglia* (Fig. 15)

Harden. Twelve to forty-eight hours in formalin-ammonium-bromide solution (c).
Bromurate. Heat block in fresh hardening solution (c) ten minutes at 45 to 50°C.
Section. Cut frozen sections at 15 to 20μ.

(1) *Wash.* Wash in two changes of distilled water. Add 10 drops of ammonia to the first water.

(2) *Impregnate.* Place sections in strong silver carbonate solution (m-1) and leave one to five minutes as determined by trial.

(3) *Wash.* Agitate gently for about fifteen seconds.

(4) *Reduce.* Plunge sections into 1 per cent formalin. Do not agitate here.

(5–8) *Wash, tone* sections as usual until gray, fix in "hypo," wash, dehydrate, clear and mount.

Result. Oligodendroglia should be stained selectively. There may be faint staining of astrocytes and microglia may be well stained.

1. First Modification of Penfield.†

(1) *Harden.* In ammonium-bromide solution two to forty-eight hours. Blocks should not be more than 3 mm. in thickness. Place blocks in 95 per cent alcohol thirty-six to forty-eight hours.

(2) *Wash.* Wash blocks about four hours to get rid of alcohol in changes of distilled water in large volume. About four times as long in the water as is taken for the block to sink to the bottom is sufficient.

(3) *Section.* Cut on freezing microtome. If alcohol is not sufficiently washed out the cutting will be difficult.

(4) *Wash.* Pass through two changes of distilled water.

(5) *Impregnate.* Leave sections in strong silver carbonate fifteen minutes to two hours as determined by trial. The most favorable time to remove sections is when they are just beginning to turn brown.

(6) *Reduce.* Plunge sections directly in 1 per cent formalin and agitate them at once. Wash, tone, fix, etc. as above.

Result. This method was successful particularly when using rabbit material. The alcohol lends a clear smooth appearance to the sections.

* Del Rio-Hortega, P. *Bol. d. l. Real Soc. Esp. d. Hist. Nat.*, 1921.

† Penfield, W. *Brain*, 1924, xlvii, 430.

Microglia cells are often stained as well as oligodendroglia. The method, like the method of Hortega, is variable, although the results are brilliant when successful. It is suitable chiefly for experimental material.

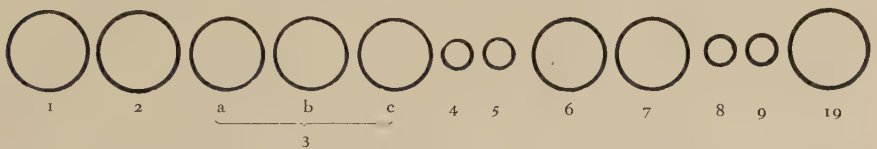

FIG. 16. Penfield's combined oligodendroglia and microglia method. 1. Ammonia. 2. Hydrobromic acid, 5 per cent. 3 a, b, c. Water. 4. Sodium carbonate, 5 per cent. 5. Silver carbonate (weak). 6. Formol, 1 per cent. 7. Water. 8. Gold. 9. "Hypo." 10. Water.

We have found that oligodendroglia can be stained in every case without fail if the above modification be employed omitting the immersion of blocks in alcohol and proceeding as follows: Inject the formalin-ammonium-bromide into the internal carotid. Remove a block from the hardening solution at the end of two hours and run sections through. If not successful repeat the following morning, and if necessary repeat again after twelve to twenty-four hours, removing each time a fresh block from the fixative. The duration of fixation seems to be of the utmost importance but is difficult to standardize.

The following modification we would recommend for routine use, as it gives uniform results and may be applied to formalin or formalin-ammonium-bromide fixed material even after a considerable period of time:

2. Second Modification of Penfield (Fig. 16). *The Combined Oligodendroglia and Microglia Method.**

Harden. Tissue in formalin or formalin-ammonium-bromide solution for an indefinite period. About a week in formalin gives excellent results.

Section. Cut sections at 20 μ on the freezing microtome and receive them in 1 per cent formalin or distilled water.

(1) *Deformalinize.* Place sections in dish of distilled water to which 10 to 15 drops of strong ammonia have been added and cover so as to prevent escape of ammonia. Leave here overnight to remove formalin.

(2) *Bromurate.* Transfer sections directly to Globus' hydrobromic acid in 5 per cent solution (5 c.c. of 40 per cent hydrobromic acid plus 95 c.c. distilled water). Place in incubator at 38°C. for one hour.

(3) *Wash.* Pass through three changes of water (a, b, c).

(4) *Mordant.* Place sections in 5 per cent solution of sodium carbonate for one hour. (Sections may remain here five to six hours without ill effect.)

(5) *Impregnate.* Pass sections with or without washing direct to Hortega's silver carbonate, weak solution (m-2) and leave them here three to five minutes or until they turn a smooth gray when transferred to the reducer. Control by taking out a section at intervals of one to two minutes and examining under the microscope. At times good results are obtained by leaving the sections in the silver solutions until they turn a light brown.

(6) *Reduce.* Place in 1 per cent formalin and agitate.

* Penfield, W. *Am. J. Path.*, 1927.

(7) *Wash.* Distilled water.

(8) *Tone.* Leave in gold chloride (1 to 500) at room temperature until sections are a smooth bluish gray.

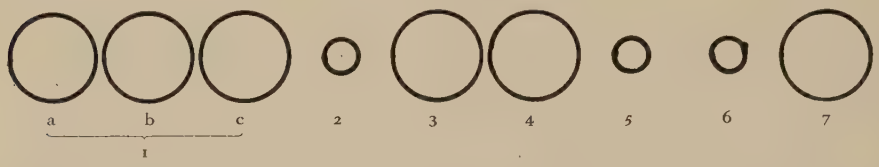

FIG. 17. Del Rio-Hortega's silver carbonate method for microglia. 1a. Water. 1b. Water, ammonia. 1c. Water. 2. Silver carbonate (weak). 3. Formol, 1 per cent. 4. Water. 5. Gold. 6. "Hypo." 7. Water.

(9) *Fix.* Hyposulphite of soda (5 per cent) as usual.

(10) *Wash.* dehydrate, clear and mount.

By this method both microglia and oligodendroglia may be stained with a considerable degree of consistency. The morphological differences of the two types of cells make it quite easy to distinguish them. This differentiation is even more easy when either type of cell has undergone some pathological change. There may be at times faint staining of astrocytes particularly if the sections are left too long in silver.

D. MICROGLIA

(Mesoglia, Hortega cells, Hortega's third element.)

VI. Del Rio-Hortega's Silver Carbonate Method for Microglia* (p. 387)

The Method† (Fig. 17).

Harden. Blocks about 3 mm. in thickness should be hardened in formalin-ammonium-bromide solution (c) for two to three days at room temperature.

Bromurate. Place blocks in fresh formalin-ammonium-bromide in a small covered glass dish and heat to about 50°C. for ten minutes.

Section. Cut sections at once in the freezing microtome at 20 to 25 μ, receiving the sections in distilled water.

(1) *Wash.* Pass sections through three changes of distilled water (a, b, c, Fig. 19) adding to the second change 4 or 5 drops of ammonia to remove the formalin.

(2) *Impregnate.* Place four or five sections in Hortega's silver carbonate "weak solution" (m-2).

(3) *Reduce.* Remove one from the silver at the end of twenty seconds and plunge directly into 1 per cent formalin. Agitate the section at once by blowing briskly on the surface of the liquid. Remove a second at forty-five seconds and another at two minutes. The sections should promptly change to smooth gray color. One minute is long enough in

* Del Rio-Hortega, P. and Asua, F. J. de. *Arch. d. cardiol. y hematol.*, 1921, ii, 161.

† The method is first given as outlined in the cited articles, and with certain minor additions which were in use in the Laboratorio de Histopatologia de la Juntu para Ampliacion de Estudios in the year 1924 when it was the privilege of one of us (W. P.) to work with the author of this method. It is a pleasure to acknowledge our indebtedness to this master of histological technique.

the reducer. If sections are brownish the duration in silver has probably been too long and neuroglia astrocytes will be found stained as well as microglia.

It is well to float sections on a slip and examine under the microscope. The microglia cells are seen as small black spider shapes. If neuroglia astrocytes are stained they are likely to be tan and, of course, larger. When the optimum duration of silver impregnation has been determined, ten to twenty sections at a time may be carried through it and into formalin with a similar result.

(4) *Wash.* Distilled water.

(5) *Tone.* Leave the sections in gold chloride solution (h) until they become an even gray with no yellow shade. If left until they are slightly purple the stain is intensified.

(6) *Fix.* Place in hyposulphite of soda (5 per cent) about a minute until they are flexible.

(7) *Wash.* Place sections in water. They may be preserved here or better in 1 per cent formalin for long periods. They may be counterstained if desired before continuing to dehydrate; clear and mount in Canada balsam. For the general background and connective tissue picro-fuchsin solution as for Van Gieson's method may be used, or picro-indigo stain of Cajal.

If the procedure is not successful place the sections in absolute alcohol for about a half hour, then wash, place in silver solution and proceed as usual. Anhydrous sodium sulphite, a pinch in 50 c.c. of water, may be used in a similar empirical fashion with occasional resultant improvement. Finally, the preliminary use of strong ammonia or strong pyridine or a mixture of the two with water in equal parts may influence the outcome favorably.

Better results are occasionally obtained by heating the silver bath to 50°C. and leaving sections here until they are of an amber color. If the solution becomes turbid before this color has been reached they have been improperly washed. Sections are next washed rapidly in distilled water before reduction. This variation is particularly helpful when working with the cerebrum of dogs according to Gallego. We have found this alternative also occasionally successful in old formalinized material.

Failure may be due to imperfectly distilled water, to over-formalinization or unsuitable formalin and to impurity in the sodium carbonate from which the silver carbonate is made. If the material is from the rabbit's brain a successful result is almost invariable. With mouse, cat, dog and human material the method gives variable results. Morphologically microglia seems to be exactly the same in all these species.

1. Del Rio-Hortega's 1927 Method (Fig. 18). More recently Del Rio-Hortega has given rules for the application of the method to macrophages as seen throughout the body in various tissues as well as to microglia.* The most generally applicable variant is as follows:

Harden. Blocks in formalin-ammonium-bromide for two to eight days. (Results may also be good if fresh material is fixed for a short time in formalin or for a long period in formalin-ammonium-bromide. For macrophages in general 10 per cent formalin is preferable.)

Section. Frozen sections at 20 μ, or imbed in gelatin if preferred.

* Del Rio-Hortega, P. *Bol. d. l. Real Soc. Esp. d. Hist. Nat.*, 1927, xxvii, 199.

(1) *Preparation.* Place sections ten minutes or more in a mixture of equal parts of pyridine, ammonia and distilled water (5 per cent crystallized sodium sulphite may be substituted for this mixture).

FIG. 18. Del Rio-Hortega's 1927 method for microglia. 1. Pyridine, ammonia, water. 2. Water. 3a, b, c. Undiluted silver carbonate. 4. Water. 5. 1 per cent formol. 6. Water. 7 Gold. 8. "Hypo." 9. Water.

(2) *Wash.* This may be omitted.

(3) *Impregnation.* Undiluted silver carbonate (m-3) is placed in three small glass dishes and the sections are passed through them in series, remaining thirty seconds in the first, one minute in the second and one minute or more in the third, depending on the temperature. Trial sections may be run through as in the original technique.

(4) *Wash.* Wash rapidly (ten to fifteen seconds) in distilled water. At times this is unnecessary. Too much washing renders the staining granular.

(5) *Reduce.* Pass to 1 per cent formalin, agitating the sections gently. Sometimes moving the sections in the reducer in this way may be found detrimental, as the silver is washed out from the macrophages. The same thing may happen with the preliminary washing.

(6, 7, 8, 9). *Wash, tone, wash* and *fix* as above.

With this method Hortega has secured good results in staining microglia and also epithelioid cells, cells of Langerhans, Kupffer cells of the liver and in general the cells included with the reticulo-endothelial system of the kidney, the tonsil, the lymphatics, lungs, etc.

Particularly good results with cat material were obtained by Hortega when he left the sections ten to fifteen minutes in undiluted silver carbonate solution and then placed the sections one by one in water where they must lie extended but without movement for about five minutes until the sections take on a pale yellow color. Reduce in 5 per cent formalin and complete as usual.

2. Modification of Cajal. *Silver Oxide Method.* Cajal has proposed a modification of this method. He substituted Bielschowsky's silver oxide solution (solution j) for the silver carbonate solution. He reports success with old formalin material, particularly pathological human material. In an earlier publication* Cajal described a similar method which also resembles that of his pupil Del Rio-Hortega under the name of "A Modification of the Method of Bielschowsky." We have used Bielschowsky's silver as recommended by Cajal but have always found it less satisfactory than Hortega's silver carbonate. The more recent description of Cajal's modification is as follows:

* Cajal, S. Ramon y. *Trav. d. Lab. d. Recb. Biol. d. l. Univ. d. Madrid,* 1925, xxiii, 157.

(1) *Harden.* Blocks as usual in formalin-ammonium-bromide solution, human material for thirty to forty-five days, animal material less than four days.

(2) *Mordant.* The sections are placed in reinforcer (i) in incubator a few hours.

(3) *Wash* rapidly three times.

(4) *Impregnate.* Place in silver oxide solution (j) diluted with an equal amount of water to which pyridine has been added. Leave here until they take on a pale straw color or heat until they become the color of "weathered straw."

(5) *Reduce.* In 5 per cent formalin and complete as usual.

E. SPECIAL METHODS

1. Modification of Del Rio-Hortega for Perivascular Glia of Andriezen.* The perivascular glia of Andriezen are astrocytes of both the fibrous and the protoplasmic types which are closely applied by their body cytoplasm to vascular adventitia while the expansions radiate off into the surrounding tissue at a distance. These cells, which were well described by Andriezen, curiously enough seem to have staining affinities similar to those of oligodendroglia and microglia.

Del Rio-Hortega has pointed out that the perivascular glia is well stained by the following technique:

The tissue should be fresh and preferably hardened not more than a day or two. Wash frozen sections from formalin-ammonium-bromide fixed material ten to twenty minutes in pure pyridine. Wash in two changes of distilled water without eliminating all the pyridine.

The sections may then be stained according to the first method of Hortega for microglia (p. 380) or the alcohol modification of Penfield for oligodendroglia (p. 378).

2. Modification of Del Rio-Hortega for Neuroglia Pigment. This method also demonstrates the pigment if present in nerve-cells and in microglia. It shows the pigment present both under normal conditions and as the result of pathological processes.

(1) *Harden.* In formalin or formalin-ammonium-bromide for an indefinite period.

(2) *Bromurate.* Heat the blocks in formalin-ammonium-bromide ten minutes at 45 to 50°C.

(3) *Section.* On freezing microtome.

(4) *Wash.* In strongly ammoniacal water.

(5) *Prepare.* Sections in sodium sulphite, 5 per cent solution for some minutes.

(6) *Impregnate.* Plunge sections directly into weak silver carbonate solution (m-2) and heat to 50°C. until sections are the color of tobacco.

(7) *Wash.* In water for one-half to one minute.

(8) *Wash.* In 95 per cent alcohol one-half minute.

(9) *Reduce.* In 1 per cent formalin.

(10) *Tone.* In gold chloride and reinforce by heating.

(11) *Fix.* In "hypo" 5 per cent.

Wash and mount as usual.

3. Del Rio-Hortega's Methods for Gliosomes and Mitochondria. Gliosomes are small rounded granules found in neuroglia (both astrocytes

* Del Rio-Hortega, P. *Bol. d. l. Real Soc. Esp. d. Hist. Nat.*, 1925, xxv, 184.

and oligodendroglia). They can be distinguished at times according to Hortega* from mitochondria; at other times it is impossible to make any such distinction. The whole question of the nature of the specific granules found in these cells must be left an open one for the present. It has been thought (Nageotte, Mawas, et al.) that these granules correspond to secretion granules and that neuroglia as a whole is a huge gland of internal secretion situated in a diffuse fashion throughout the central nervous system. The method may be carried out in one of three ways.

(a) The Method. Staining in the Cold (Mitochondria).†

(1) *Harden.* Place blocks of 2 to 3 mm. thickness in formalin-iron-alum solution (n).

(2) *Section.* Cut sections on freezing microtome at about 10μ, taking care not to freeze the tissue too hard.

(3) *Wash.* Pass through two good-sized dishes of water. Add 5 to 6 drops of ammonia to the first water to render the sections more flexible.

(4) *Impregnate.* Silver carbonate solution (m-2) for about five minutes, depending on the temperature of the room.

(5) *Wash.* Distilled water for fifteen to thirty seconds. Do not move sections too much here. This is an important step. The water acts as a differentiator removing silver from the general tissue and leaving it only in the mitochondria. If this is not done carefully only the more voluminous and more energetically impregnated gliosomes are likely to be stained.

(6) *Reduce.* Formalin (1 to 200). For the study of mitochondria the section should turn a grayish color. Gliosomes are best seen in sections which take on a reddish hue. Wash.

(7) *Tone.* Leave sections in 1 to 500 gold chloride solution for about fifteen minutes or heat gently until the color is a little more intense.

(8) *Fix.* In "hypo" as usual.

Result. At least in dog and cat material the mitochondria of neuroglia are well stained as are sometimes similar granules in nerve-cells.

(b) Staining in the Heat (Gliosomes).

(1) *Harden.* Two to eight days in the formalin-iron-alum solution. (Tissue fixed for months in formalin-ammonium-bromide may be used if the sections are left twenty-four hours in 5 per cent solution of iron alum.)

(2) *Wash.* Two or three large dishes of distilled water to which ammonia is added followed by a wash in water without ammonia.

(3) *Impregnation.* Heat in silver carbonate (m-2) to which 2 or 3 drops of pyridine have been added at 45 to 50°C. shaking occasionally, until sections take on a light tobacco color.

(4) *Wash.* One to three minutes in distilled water.

(5) *Reduce.* Formalin (10 per cent).

(6) *Tone.* Fifteen minutes in the cold and then intensify the color by heating gently.

(7) *Fix.* As usual.

* Del Rio-Hortega, P. *Bol. d. l. Real Soc. Esp. d. Hist. Nat.*, 1925, xxv, 34.

† For procedure (*a*) and (*c*) the formalin uranium (solution o) may be used. This fixative and mordant gives particularly good results for protoplasmic astrocytes and even better for oligodendroglia.

Result. This procedure is used chiefly to stain gliosomes. The advantage of this modification is that neuroglia bodies and expansions are stained without blotting out the granules.

It is stated that a reducer of 5 to 10 per cent formalin demonstrates gliosomes by preference; 1 per cent reducer tends to demonstrate the mitochondria of glia and in part those of nerve-cells; dilution of reducer to 1 to 400 produces splendid staining of mitochondria in neuron bodies and larger axons. For better staining of gliosomes in oligodendroglia dilute the silver solution and add about 20 drops of 95 per cent alcohol as well as pyridine to it.

(*c*) *Double Impregnation.*

(1) *Harden.* In formalin-iron-alum solution or in this solution plus 2 per cent of ammonium-bromide, three to four days.
(2) *Section* and *wash* as in (*b*).
(3) *Prepare.* Place sections in 2 per cent silver nitrate and heat gently.
(4) *Wash.* Rapidly.
(5) *Impregnate.* Weak silver carbonate one minute.
(6) *Wash.* Very rapidly.
(7) *Reduce.* Formalin 1 per cent.
(8) *Tone* and *fix* as in (*b*).

Result. This procedure succeeds at times when (*a*) fails and is particularly helpful in staining the cerebellar neuroglial cells of Bergmann, if formalin-uranium is used to harden the tissue. The procedure also demonstrates very well the granules of ependymal cells.

4. Counterstaining of Microglia. For staining of lipoid material G. Herxheimer's stain may be used. This is a saturated solution of scarlet red in 70 per cent alcohol and pure acetone. Staining takes place very quickly.*

For staining iron in macrophages Del Rio-Hortega follows the procedure outlined by Perls as follows:

Counterstain of Microglia for Iron (Perls).

(1) After staining the sections with silver and toning, gently heat them to 45 or 50°C. in a solution of 5 per cent potassium ferrocyanide for fifteen minutes.
(2) Place them in hydrochloric acid (10 per cent) at a temperature of about 25 to 30°C. for fifteen minutes.
(3) Wash well in two changes.
(4) Stain with Ziehl's fuchsin fifteen seconds.
(5) Wash well in water.
(6) Place in saturated solution of picric acid and leave there some seconds until sections darken.
(7) Differentiate in 95 per cent alcohol until sections become pale red.
(8) Clear in carbol-xylol-creosote (f).
(9) Mount in balsam.

* Mallory, F. B. and Wright, J. H. Pathological Technique, Phila., 1924.

When microglia contains much iron, it will be demonstrated well out in the expansions. In their ameboid forms a large amount of iron will be found in these cells.

F. SOLUTIONS

a. Distilled Water. It is our custom to use doubly distilled water for all solutions and washings, the second distillation being done in glass. In the laboratories of Madrid the taps supply water straight from the melting snows of the Guadarramas. This is not distilled for routine use.

b. Ten Per Cent Formalin.

Merck's blue label 40 per cent formaldehyde	10 c.c.
Distilled water	90 c.c.

We have not found it necessary to add chalk to this formalin* as it is very nearly neutral. When using other makes of formol we have added chalk to the stock. We believe that Cajal uses formalin prepared by Merck or Kahlbaum to which he adds chalk. It is the custom of Del Rio-Hortega to use Merck's formalin. Highly acid formalin is prejudicial to many of the reactions.

c. Cajal's Formalin-ammonium-bromide Solution (F.A.B.).

Formalin (Merck's blue label, 40 per cent)	14 c.c.
Ammonium bromide	2 gm.
Distilled water	86 c.c.

d. Cajal's Gold Chloride and Sublimate Solution. Prepare fresh bath each time with scrupulously clean glassware as follows:

Mercury bichloride crystals	0.5 gm.
Gold chloride (Brown, Merck) 1 per cent	10 c.c.
Distilled water	50 c.c.

Pulverize the sublimate and add it to the 50 c.c. of water and place over alcohol flame so as to dissolve rapidly but do not overheat, or place in hot water bath. When dissolved add gold chloride to solution which is still hot. Filter the mixture. Cajal has warned against buying the powdered preparation of sublimate. The gold chloride keeps well in a brown bottle for long periods. Cajal advised in his last formula a smaller concentration of gold than here given, i. e., 6 c.c.

e. Cajal Fixing Bath.

Sodium hyposulphite	5 gm.
Water	70 c.c.
Alcohol (95 per cent)	30 c.c.
Concentrated solution of sodium bisulphite	5 c.c.

* The pH of the Merck's blue label 40 per cent formalin has been about 6.8. The 10 per cent solution made from this has had an average pH of 6.2.

f. Carbol-xylol-creosote Mixture.

Creosote	10 c.c.
Phenol	10 c.c.
Xylol	80 c.c.

g. Del Rio-Hortega's Silver Carbonate Solution (Lithium) for Astrocytes.

Silver nitrate (10 per cent)	5 c.c.
Lithium carbonate (saturated solution)	20 c.c.
Ammonium hydroxide q.s. to dissolve precipitate	
Distilled water ad	75 c.c.
Filter and keep in a brown bottle	

When the first two chemicals are combined silver carbonate comes down as a voluminous precipitate. The ammonia should then be added drop by drop stirring all the while until the precipitate disappears and ammoniacal silver goes into solution. Care must be taken to add no more ammonia than just enough to cause this resolution. A small amount of black dustlike precipitate will remain undissolved and may be filtered off. The solution may usually be kept for long periods.

This is the preparation employed by Del Rio-Hortega more recently. In the original publication of the method (p. 374) Del Rio-Hortega recommended silver nitrate (10 per cent) to which was added an equal or larger amount of saturated solution of lithium carbonate. He then decanted the liquid and washed the precipitate with 50 c.c. of distilled water. He decanted again and then added 15 to 20 c.c. water and followed with ammonia sufficient to dissolve the precipitate, after which water was added so as to increase the total volume to 50 c.c.

b. Gold Chloride Solution for Toning.

Gold chloride (yellow)	1 gm.
Distilled water	500 c.c.

It is not necessary to use the more expensive brown gold for toning; in fact the yellow variety seems to serve the purpose better.

i. Cajal's Reinforcer.

Neutral formalin	30 c.c.
Ammonium bromide	3 gm.
Distilled water	70 c.c.

j. Bielschowsky's Ammoniacal Silver Bath as Prepared by Cajal.

Silver nitrate, 1 per cent	20 c.c.
Sodium hydroxide, 40 per cent	22 drops

Wash the precipitate with distilled water six times.
Add to the precipitate at a temperature of 22°c.

Distilled water	110 c.c.
Ammonium hydroxide	4 c.c.

Stir until precipitate is dissolved. Keep in dark bottles. The test for the activity of this silver oxide solution is the rapidity with which sections turn dark brown when placed in it at room temperature.

k. Bielschowsky's Ammoniacal Silver Bath as Prepared by Del Rio-Hortega for Achúcarro's Method.

Silver nitrate 10 per cent	30 c.c.
Sodium hydroxide	40 drops

Wash the precipitate ten or twelve times in distilled water, using a liter at least. Add to the precipitate 50 c.c. of distilled water. Add to this ammonia in just sufficient amount to dissolve the precipitate, stirring without too great violence. Add no more ammonia after the solution smells of it. Add distilled water so as to bring total up to 150 c.c.

l. Mordant for Hortega's Fourth Variant.

Tannin	3 gm.
Ammonium bromide	1 gm.
Distilled water	100 c.c.

m. Del Rio-Hortega's Ammoniacal Silver Carbonate Solution.

1. Strong solution.

Solution of silver nitrate (Merck) 10 per cent	5 c.c.
Solution of sodium carbonate (pure) 5 per cent	20 c.c.
Ammonium hydroxide (sufficient to dissolve precipitate)	
Distilled water up to	45 c.c.

The ammonium hydroxide, as indicated above, should be added drop by drop until the precipitate is just dissolved, stirring the solution all the while. Finally, filter and place in a dark bottle, where it will keep for long periods.

2. Weak solution. Is prepared in the same way, except that distilled water is added up to 75 c.c.

3. Undiluted silver carbonate is made up the same way but no water is added after the precipitate is dissolved with ammonia.

n. Del Rio-Hortega's Formalin-iron-alum Solution.

Formalin (40 per cent)	10 c.c.
Distilled water	90 c.c.
Iron alum (pure)	6 to 8 gm.
Filter	

o. Del Rio-Hortega's Formalin-uranium Solution.

Formalin (40 per cent)	10 c.c.
Uranium nitrate	1 to 2 gm.
Distilled water	90 c.c.

CHAPTER VIII

PROTOZOOLOGICAL METHODS

D. H. Wenrich

Introduction 389. Examination of living protozoa 389. Temporary killing and staining methods 395. Permanent mounts 396.

I. Introduction

In order to gain a full knowledge of any particular kind of Protozoa it is necessary to study them in the living condition in as normal an environment as possible, and, in addition, to employ a wide range of technical aids. In the present section suggestions are offered for (1) the collection and examination of living Protozoa, (2) intravitam staining, (3) temporary methods of killing and staining and (4) preparation of permanent mounts. As a section of a general work, the presentation is not intended to be exhaustive. Those methods which seem to the author to be of the greatest usefulness have been selected. Some familiarity with laboratory methods is presupposed.

II. Examination of Living Protozoa

1. Free-living Protozoa. *a. Collection.* Collection of free-living Protozoa may be done by simply dipping up a quantity of water from a stream, pool or larger body of water, or by using some form of special apparatus.* Where variety of species is desired, it should be remembered that Protozoa are to be found in moist soil, on and in the bottom deposits of water bodies and attached to all kinds of submerged objects, including growing water plants as well as swimmng freely in the water, and collections from all these sources should be made. If the sample collected is to be representative of a body of water, such as a reservoir, then it should be taken out away from shore and away from any special environmental condition such as floating objects.

b. Concentration Methods. These are frequently employed, usually involving some kind of filtering device. The simplest method is the following: Pass the sample of water through filter paper in an ordinary funnel. When 10 to 20 c.c. of water remain in the funnel quickly pour the residue into a vessel and examine as a concentrate. By measuring the original sample and counting all the individual specimens of each kind in the concentrate, this method can be made quantitative. Ordinary filter paper will permit many of the smaller forms to pass through. A hard filter paper may be used, but filtering is slowed down and the funnel will need to be agitated

*Whipple, G. C. The Microscopy of Drinking Water. Ed. 4. N. Y., 1927.
Ward, H. B. and G. C. Whipple. Freshwater Biology. N. Y., 1918.

frequently to prevent the Protozoa from becoming attached to the paper. A suction exhaust will hasten filtering but may injure the more delicate species.

Concentration in the Field. Select a wide-mouthed bottle or jar provided with a stopper having two holes through it. In one hole insert the stem of a small funnel in an inverted position with its broad end covered with bolting cloth. In the other hole in the stopper insert a larger funnel in an upright position. Put the stopper in place and pour the water into the upright funnel. When the bottle is full the water will filter out through the smaller funnel, the organisms being retained by the bolting cloth. A relatively large volume of water may thus be concentrated for transportation to the laboratory.*

Towing with a tow net is essentially a method of concentration. By using certain forms of tow net and observing certain rules, this method may be made quantitative.†

The Sedgwick-Rafter method is designed to give quantitative results. Special apparatus consists, first, of a graduated funnel of 500 c.c. capacity which is cylindrical throughout most of its length. Near the lower end it tapers to a much narrower cylinder which is also graduated. The lower end is provided with a perforated rubber stopper into the opening of which is inserted a glass tube up to the upper surface of the stopper. At the lower end of the glass tube a rubber tube provided with a clamp is attached. The upper end of the stopper is covered with a disk of bolting cloth and on this enough fine sand is poured to make a layer of $\frac{3}{4}$ to $1\frac{1}{2}$ inch in depth. The sand should be fine enough to pass through a sieve having 60 meshes to the inch. Two other special pieces of apparatus are (1) the counting cell, which is a microscopical slide with a brass rim cemented to it to make a cell exactly 1 mm. deep, and (2) the ocular disk, which has a large square on it ruled into 100 smaller squares. This disk is placed in the ocular of the microscope and such a combination of lenses and tube length is chosen that one side of the large square corresponds to 1 mm. on the stage of the microscope. (Calibrate with a stage micrometer.) When the counting cell is in place, by counting all the organisms within the large square of the ocular disk, one actually counts the number in a cubic millimeter, since the cell is just 1 mm. deep. The apparatus is used in the following manner:

Having secured a carefully collected sample of water, stir it to obtain an even distribution of the organisms, pour a little water into the funnel to wet the sand and fill the funnel up to the 500 c.c. mark. Next remove the clamp from the outlet tube and allow filtering to proceed. When a small quantity of water, say 1 c.c. remains in the lower end of the funnel, replace the clamp to stop the filtering. Place a tumbler or beaker under the funnel and remove the stopper, allowing the sand and concentrate to fall into the tumbler. Quickly wash the inside of the funnel with a small measured quantity (say 9 c.c.) of sterile water to remove attached organisms. Next gently stir the concentrate and sand to

* Hall, W. E. *J. Roy. Micr. Soc.*, 1924, 46.

† *See* Ward and Whipple. *Loc. cit.* p. 389.

free the organisms from the sand, and after letting it stand for a few seconds so that the sand may settle to the bottom, decant the water containing the organisms into another container. Again stirring the concentrate to secure even distribution of the organisms, take some of it up with a pipette and place it in the counting cell.

To prevent overflowing place the cover glass over most of the cell before the concentrate is added. When the cell is full adjust the cover glass and place the cell on the stage of the microscope. Using a mechanical stage to prevent duplication of fields, count all the organisms of each kind in a definite number of ocular squares, each in a different field of the microscope, and record the numbers. The number of individuals in each square is really the number in cubic millimeters of the concentrate. The results are expressed in terms of the number of each kind of organism per cubic centimeter of the original sample. In order to bring the actual counts into this form the following formula is used:

$$N = \frac{t}{n} \times \frac{1000\ c.}{v}$$

in which N is the number per cubic centimeter of original sample, t is the total number of a particular kind counted in all the squares, n is the number of squares counted, c is the number of cubic centimeters of the concentrate after washing the funnel and v is the number of cubic centimeters of the original sample. If we suppose that 20 squares were counted and that 10 c.c. was the amount of concentrate after filtering 500 c.c. of the original sample, and if we substitute these quantities in the above equation, we get

$$N = \frac{t}{20} \times \frac{1000 \times 10}{500}$$

which cancels out to $N = t$, making calculation very simple.

This calculation needs to be made for each kind of organism recorded and, in addition, a survey should be made of the entire counting cell to find any kinds that may have been missed in the series of squares counted. Such a random sampling method naturally has inherent defects, but in spite of these, tests have shown that parallel determinations from the same sample do not usually differ more than 10 per cent from each other.*

c. Examination. If the collected sample of water is allowed to stand undisturbed in a vessel for an hour or more the Protozoa tend to distribute themselves in different parts of the vessel depending on their several tropic responses. Those that are positively phototropic will be found on the most brightly illuminated side. Geotropism will send some to the top and others to the bottom. If a random sampling method is used to obtain quantitative results, then the sample or concentrate should be thoroughly stirred before examination.

In ordinary work, proceed as follows: place a drop of water containing the organisms on a slip and cover with a cover glass. A depression slide may be used but is not necessary. If there are no objects in the drop capable of supporting the cover glass, add pieces of fine glass rods or tubing, or pieces of cover glass to the drop, or attach small lumps of wax to the corners of a square cover glass to prevent crushing the animals. If the cover glass is unsupported, evaporation will deplete the film of water and the organisms

* Whipple, G. C. *Loc. cit.* p. 389.

will be distorted by pressure. This, however, is a decided advantage for observing certain details, such as the collecting canals of the contractile vacuoles of Paramecium. At one time it was a common practice to compress organisms in a special compressor for examination of their finer structures.

Compensate for evaporation by adding water at the edge of the cover glass with a dropper, or seal a short shell vial to one end of a slip with paraffin or cement to act as a water reservoir, and arrange a small string or coarse thread to act as a capillary siphon to carry water from the reservoir over to the fluid under the cover glass.

Examination in cultures is possible where Protozoa have been cultured in a Syracuse watch glass or similar small dish. Place the dish on the stage of the microscope and examine the animals directly. Low power objectives will not need to touch the water, but for higher magnification, water-immersion objectives are available. This method is especially advantageous for sedentary forms and for tracing development of a culture.

A hanging drop is convenient for confining one or a small number of Protozoa in a limited amount of fluid for prolonged observation. Place a small drop of the culture containing the organisms in the middle of a round cover glass, or isolate a single animal in such a drop and invert the cover over the concavity of a depression slide and seal with vaseline, paraffin, or other cementing substance, or seal a glass ring to an ordinary slip to make a chamber over which the cover with the drop is inverted and sealed. Special forms of culture slides are also to be had from the supply houses.

Slowing the movements or restricting the activities of ciliates and flagellates: Either (1) isolate in a small drop of fluid, e. g., a hanging drop, or (2) add a little cotton wool to the drop on the slide, or (3) thicken the medium by adding 2 per cent or 3 per cent gelatin, quince-seed jelly, cherry-tree gum or carragheen, or (4) add some anesthetizing agent.

Anesthetization. (1) Spray alcohol, ether or chloroform on the water, or (2) drop small crystals of menthol on the water, or (3) add one of the following aqueous solutions to the organisms on a slide or in a dish: 0.1 per cent nicotine, 0.1 per cent to 0.2 per cent chloral hydrate, 1.0 per cent cocaine hydrochlorate, 1.0 per cent cocaine hydrochloride, 1.0 per cent magnesium sulphate, or magnesium chloride, or 0.1 per cent chloretone. Cole and Richmond* state that 1 drop of 0.12 per cent chloretone added to a drop of equal size of culture containing paramecia, giving a concentration of about 0.056 per cent chloretone, will anesthetize the paramecia in about ten minutes and will keep them quiet for two days or longer up to ten days.

2. Associated Protozoa. *a. Ectozoic Protozoa.* Examine if possible while these are still attached to their hosts. When this is impossible examine them in their native medium, which is, of course, that of their hosts.

* Cole, W. H. and Richmond, E. *Proc. Soc. Exp. Biol. & Med.* 1925, xxii, 231.

b. Endozoic Protozoa. Examine in the body fluids of the hosts or in some fluid which is isosmotic, or nearly so. Examine blood-inhabiting species in the blood itself by placing a drop of blood on the slide and covering with a cover glass, or dilute the blood with some saline diluent such as physiological salt solution, Ringer's solution (to 100 c.c. of distilled water add 0.8 gm. NaCl, 0.02 gm. $CaCl_2$, 0.02 gm. KCl, 0.02 gm. $NaHCO_3$ and (optional) 0.1 gm. dextrose); Locke's solution (to 100 c.c. of distilled water add 0.9 gm. NaCl, 0.04 gm. KCl, 0.02 gm. $CaCl_2$, 0.001 to 0.003 gm. $NaHCO_3$ and 0.1 gm. dextrose), or a solution made by adding 0.5 gm. of sodium citrate and 0.3 gm. sodium chloride to 100 c.c. of distilled water. Examine tissue inhabiting species by teasing out the tissues in one of the above saline solutions.

Intestinal Protozoa. Feces or intestinal contents usually require dilution before examination. In most cases, a sodium chloride solution of 0.4 per cent to 0.6 per cent is preferable to stronger solutions, and Cleveland* states that 0.2 per cent sodium chloride is better for the Protozoa in certain tropical termites. However, for the flagellate Trichonympha, alive outside its termite host, he recommends a solution made up as follows:

Sodium chloride	0.30 gm.
Calcium chloride	0.02 gm.
Potassium chloride	0.02 gm.
Magnesium chloride	0.01 gm.
Sodium dihydrogen phosphate (NaH_2PO_4)	0.001 gm.
Sodium hydrogen carbonate ($NaHCO_3$)	0.01 gm.
Löffler's blood serum	0.50 gm.
Distilled water	100.00 c.c.

Kirby† recommended 67 per cent Locke's solution as an observation medium for other flagellates of termites. For prolonged observation of intestinal Protozoa, the cover glass should be ringed with vaseline, soft paraffin or paraffin oil to prevent evaporation. The amount of dilution should be adjusted to allow plenty of light to pass through for microscopic observation. The exact amount can best be determined by experience. It may be noted that when intestinal and other endozoic Protozoa are examined in a saline medium without ringing the cover glass, they will frequently become immobilized when they approach the edge of the cover. Such gelated specimens of ciliates and flagellates often reveal the number and character of their locomotor organs which can be made out only with difficulty in the motile animal. Examination with the dark field will aid in determining the number of flagella of flagellates.

Warm-blooded host-inhabiting protozoa often quickly lose their motility or degenerate when the temperature is reduced below that normal for the

* Cleveland, L. R. *Biol. Bull.*, 1925, xlviii, 282.

† Kirby, H. *Univ. Calif. Pub. in Zool.*, 1926, xxix, 25.

hosts. For maintaining a constant temperature, use a warm stage or place the microscope in a warm box. There are several types of warm water and electric warming stages on the market.

3. **Intravitam Staining.** Intravitam staining methods are equally applicable to free-living and associated Protozoa. For free-living forms, the dyes are usually dissolved in distilled water or in the native medium of the organisms, while for endozoic species they are dissolved in physiological salt solution. The agents used are either finely divided particles in suspension or basic dyes.

Methods for Food Vacuoles in Ciliates. Demonstrate by placing non-toxic colored particles, such as finely divided carmine or finely divided carbon ("Chinese ink" or "India ink") into the culture medium. Such colored particles are often ingested in the same way that food particles are and the food vacuoles thus become readily visible.

The acidity or alkalinity of the food vacuoles may be demonstrated by neutral red (1–50,000) which becomes a cherry-red color at the acid end and yellow toward the alkaline end of its color range, or by phenolsulphonphthalein* which is pink in alkaline and yellow in acid conditions.

Methods for Contractile Vacuoles. To see the contractile vacuoles of Paramecium discharge outside of the body, place the animals into a very dense suspension of carbon particles in a small drop of water on a slip and cover with a cover glass.† Howland‡ found that "alizarin blue (Grübler's) is a selective submortem stain for the walls of the contractile vacuole and its feeders" (Paramecium) causing them to dilate and become much more readily visible.

Methods for Other Cytoplasmic Structures. Stain mitochondria in Protozoa as in Metazoa with Janus green or Janus green B, diluting 1 to 10,000 or even 1 to 500,000.§ Use neutral red in concentrations of 1 to 10,000 or stronger to give a diffuse cytoplasmic stain or to stain various structures selectively, for example Golgi bodies in gregarines‖ and sap-filled vacuoles of Dinoflagellates.¶

Basic dyes appear to stain more readily intravitam than do acid dyes. Ball** tested a number of dyes and gives the following list with the minimum concentration that would stain the cytoplasm of Paramecium and the toxicity as indicated by the percentage of animals dead at the end of one hour:

* Shipley, P. G. and DeGaris, C. F. *Science*, 1925, lxii, 266.
† Jennings, H. S. *Zool. Anz.*, 1904, xxvii, 656.
‡ Howland, R. B. *J. Exper. Zool.*, 1924, xl, 251.
§ Causey, D. *Trans. Am. Micr. Soc.*, 1925, xliv, 156.
‖ Joyet-Lavergne, P. *Compt. Rend. Soc. de Biol.* 1926, xciv, 830.
¶ Dangeard, P. *Comp. Rend. Acad. de Sci.*, 1923, clxxvii, 978.
** Ball, G. H. *Biol. Bull.* 1927, iii, 68.

Dyes	Minimum Concentration That Will Stain Paramecium	Toxicity: Per Cent dead in one hour
Bismarck brown	1 to 150,000	0
Methylene blue	1 to 100,000	5
Methylene green	1 to 37,500	5
Neutral red	1 to 150,000	3
Toluidine blue	1 to 105,000	5
Basic fuchsin	1 to 25,000	30
Safranin	1 to 9,000	30
Aniline yellow	1 to 5,500	0
Methyl violet	1 to 500,000	20
Janus green B	1 to 180,000	40

Additional intravitam dyes useful for Protozoa are brilliant cresyl blue (1 to 50,000), Nile blue (1 to 30,000) and rhodamine (1 to 20,000);* Azur 1, indulin, and Victoria blue in concentrations of 1 to 100,000 up to 1 to 200,000;† and a new stain, "spirsil," in concentrations of 1 to 32 or 1 to 64, as recommended by Varga.‡

III. Temporary Killing and Staining Methods

Intravitam dyes, in strong concentrations, may often serve as both killing and staining agents. (1) Janus green B: add one drop of a 0.5 per cent stock solution to 10 to 20 drops of water (free-living forms) or salt solution (parasites) and either mix with the Protozoa on the slide or place it at one edge to be drawn under the cover by capillarity.§ (2) Use alcoholic solutions (saturated) of methylene blue, methyl green, gentian violet, safranin and other dyes diluted to various strengths in water in the same manner as the Janus green.‖

Writing fluids may be also employed¶ for staining classroom material (Paramecia). Place a drop of culture on a slip and stir in a small quantity, "a dab or two" of Sanford's red ink with a toothpick. Put the cover glass in place and after four or five minutes add Waterman's ink from a fountain pen at one side of the cover glass. As the Paramecia come into contact with the blue ink they discharge their trichocysts, the cytoplasm becomes red, the cilia a "flame" color and the trichocysts blue. The nucleus usually stains also. While somewhat capricious, this method gives some very interesting results.

* Becker, E. R. *Biol. Bull.*, 1926, l, 235–238.
† Rumjantzew, A. and Kedrowsky, B. *Protoplasma*, 1926, i, 189.
‡ Varga, L. *Ztschr. f. wiss. Mikr.*, 1926, xliii, 338.
§ Hogue, M. J. *Stain Technology*, 1926, i, 35.
‖ Hausman, L. A. *Am. Nat.*, 1920, liv, 333.
¶ Halter, C. R. *Science*, 1925, lx, 90.

Iodine has long been used for killing Protozoa for quick examination. (1) Lugol's solution (Potassium iodide, 6 gm., iodine, 4 gm., water, 100 c.c.) is commonly employed. Dilute this stock solution 1 to 5 or 1 to 10 with water and then mix with the organisms on the slide or add at one side of the cover glass. (2) Dilute a saturated solution of iodine in alcohol containing 3 per cent potassium iodide with water and use in the same way.*

Osmic acid, either in the form of vapor or in a 1 per cent aqueous solution is frequently used for treating Protozoa for temporary examination. (1) Invert the slide with the Protozoa on it over the mouth of a bottle or other vessel containing a 1 per cent solution of osmic acid and the vapor will kill the organisms in a few seconds, or, (2) mix a drop of the solution with the organisms on the slide. Causey† recommends the following for class use: centrifuge the culture; pour off the supernatant fluid and add a few drops of a 1 per cent solution of osmic acid so that the resulting mixture will be about a ½ per cent solution of osmic; add a little distilled water, and the material is ready for distribution to the class. If permanent mounts are desired, prolong the fixation for one-half to one hour.

Copper Salts. Treat Paramecium and other Protozoa with 1 per cent or 2 per cent aqueous solutions of copper sulphate, copper acetate or copper bichromate for temporary examination. Little or no distortion results.

Alcohol. To demonstrate the pellicle of Paramecium and other ciliates, apply a 35 per cent solution. The pellicle is raised up in blisters.

Methods for Nuclei. (1) Methyl green and acetic acid: use a strong solution in 1 per cent acetic acid. Add at one side of the cover glass on the slide and it will diffuse under, killing the organisms that it may reach and staining the nuclei. (2) Methyl green in alcohol: use a 0.5 per cent solution in 50 per cent or 70 per cent alcohol. Employed as the acetic acid mixture this sometimes gives better results. (3) Aceto-carmine (p. 468), use as the methyl green solutions. It kills and provides a selective nuclear stain.

IV. Permanent Mounts

1. General Section. *a. General Directions.* The methods for making permanent mounts of any kind of Protozoa need to be adapted to the nature of the organisms, their habitat, and the special results desired. In general, it is not advisable to limit oneself to any one method of procedure. Different methods reveal different structures and where a full knowledge is desired, as many varied methods need to be employed as will reveal the greatest number of structural details.

Surface Scums of Cultures. If the culture bears a surface scum in which the Protozoa may be more or less entangled, drop a clean cover glass on the scum, then lift it up with forceps, keeping it horizontal, and a section of the scum will adhere to the under surface. Tilt the cover and drain off

* Hausman, L. A., *loc. cit.* p. 395.

† Causey, D. *Science*, 1925, lxii, 113.

excess water on a piece of filter paper, then drop, scum side down, on the surface of the fixative which has been placed in a convenient container. Invert the cover in the fixative, bringing the scum side up, before it can drop to the bottom of the container and handle in this position until it is cleared and ready for mounting on a slip.

Albumen Smears. There are two ways of préparing albumen smears of free-living forms. (1) Smear a slip or cover with egg albumen, either fresh or in the form of Mayer's albumen fixative, and place a drop of the culture on this smear. Allow the culture fluid to evaporate until there is only a thin film. Then drop the smear, film side down, on the fixative and handle as indicated above. (2) Fix the organisms by pipetting them into the fixative in a test tube or bottle. When they have settled to the bottom decant or pipette off the fixative and pour in the washing fluid. By successively allowing the organisms to settle, or centrifuging, then decanting and pouring in the next fluid, carry the fixed specimens through graded alcohols to 80 per cent or 85 per cent alcohol, and then affix to a slip or cover as follows: Smear the slip or cover with a thin layer of albumen, then pipette a small drop of the alcohol containing the organisms on to the smeared surface. The alcohol spreads rapidly, distributing the contained specimens, and coagulates the albumen. Allow to evaporate a little, but before drying can occur, place the slide or cover in a dish of 80 per cent or higher strength of alcohol for further hardening of the albumen, after which the specimen is handled as a whole object, as indicated previously.

Handling in Bulk. Instead of making albumen smears, leave the organisms in the tubes or vials in which they are fixed through the entire series of steps up to clearing, concentrating at the bottom for each change of fluid either by sedimentation or by centrifuging. Mounting requires care. It is best to add thick balsam to the specimens in their container. A small drop of the thick balsam containing specimens is placed on a slip, then a drop of thinner balsam placed on this, and the cover glass applied. The thinner balsam will spread to the outer edge of the cover, and the organisms will stay nearer the middle. If the number of specimens is small it is best to transfer them in xylol to a watch glass and carry out the further steps under a dissecting binocular microscope. For the larger Protozoa it is well to support the cover with pieces of cover glass or fine glass rods.

Fixing and Staining on a Slip. Place a drop of water or culture containing the organisms on a slip and before the cover glass is applied, place a white cotton thread slightly longer than the width of the cover glass across the slide in one edge of the drop. Place an additional bit of thread on each side of the drop to keep the cover level and put the cover in place. If, on examination, it is desired to fix and stain the organisms present, apply a blotter or other absorbent paper to the side of the preparation nearest the long piece of thread, to take up any excess water, then apply the fixative at the opposite side and draw under by withdrawing fluid with

the blotter on the side with the thread. The entire series of processes of fixation, washing, staining, dehydration, clearing and mounting can be carried out without moving the cover. Clear with clove oil or similar oil, after 95 per cent or absolute alcohol, and follow with xylol and then balsam.*

Washing, Dehydration, Etc. After fixatives containing alcohol, wash out in 50 per cent or 70 per cent alcohol. Wash out aqueous fixatives with water. It is customary to put iodine in one of the washing fluids if the fixative has contained mercuric chloride. The writer finds that the iodine treatment of smears can be dispensed with if the washing can be prolonged for twenty-four hours or more, and the washing fluids changed several times. If the time of washing is to be short, the iodine treatment is advised, usually in the form of Lugol's solution added to the 70 per cent alcohol, 1 c.c. or 2 c.c. to 100 c.c. of the alcohol. Extra hardening in alcohols higher than 70 per cent is unnecessary with most of the fixatives mentioned below.

Downward steps from higher alcohols to water are usually accompanied by strong diffusion currents unless the changes are gradual. If it is inconvenient to employ the drop method (p. 188), smears may be passed through the following grades of alcohol: 70 per cent, to 50 per cent, to 30 per cent, to 10 per cent, to water, to stain. One to five minutes in each alcoholic solution is usually sufficient. After staining and washing, fewer steps are necessary for dehydration: e. g., water, 30 per cent, to 70 per cent, to 95 per cent, to absolute alcohol or a clearing oil, to xylol, to balsam. Damar may be substituted for Canada balsam and is preferred by many workers (p. 141).

Mounting Individual Specimens. Bowen's method.† Transfer the stained organisms from alcohol to ether-alcohol for one to several hours, then into thin collodion for twenty-four hours or less. Pour the collodion with the organisms into a flat dish and separate the individuals with a needle or probe. When partly hardened by evaporation, complete the hardening with chloroform vapor. Remove the thin sheet of collodion and cut into blocks, each containing a single specimen. These blocks can then be cleared in cedar wood oil and mounted in balsam.

Mueller's Method.‡ Transfer individual specimens from 95 per cent alcohol to a drop of euparal on a slip. Warm over an alcohol lamp or electric bulb and orient with a needle. Allow this to harden for a week out of reach of dust then add a little fresh euparal and a cover glass. The hardened layer of euparal prevents any injury to the specimen in case the cover glass is broken. This method is especially advantageous for class room material.

b. Fixation. For any particular kind of Protozoa, the best fixatives can be determined only by experiment. In most cases a number of different fixatives need to be employed to bring out the different structural details.

* Tozer, E. *J. Roy. Soc.*, 1909, p. 24.

† Bowen, W. K. *Trans. Am. Micr. Soc.*, 1923, xlii, 156.

‡ Mueller, J. F. *Trans. Am. Micr. Soc.*, 1926, xlv, 54.

For example, in various species of Trichomonas it has been found that Janicki's parabasal body will not stain after Schaudinn's fixative, but will stain after chromic acid or osmic acid mixtures without acetic acid. Most of the standard cytological and histological fixatives can be used for Protozoa, but there are some which have come to be regarded as especially valuable for this group. Most of these are best used warm, and the fixation will be completed in a few minutes to an hour unless some special method like that for Golgi bodies is being used. When one is dealing with a piece of tissue, an hour or longer should be allowed.

Sublimate Mixtures. Schaudinn's fluid was originally made by taking 2 parts of a saturated aqueous solution of mercuric chloride and adding 1 part of absolute alcohol. Maier* took 200 c.c. of distilled water and added 1.2 gm. of sodium chloride, 10 gm. of mercuric bichloride, and 100 c.c. of absolute alcohol. Nowlin† makes Schaudinn's by adding 20 parts of absolute alcohol to 80 parts of a saturated solution of mercuric bichloride. Most workers add from 1 to 5 parts of glacial acetic acid before using. Since Schaudinn's fluid has become so generally employed for Protozoa it is recommended as a standard routine fixative, but for any complete investigation it needs to be supplemented by others.

Other sublimate mixtures which give results comparable to those given by Schaudinn's fluid are (1) sublimate-acetic (p. 421) and (2) Worcester's fluid (p. 420). These and Schaudinn's fluid are best used hot. In a series of of tests made for the writer by Scott, the best fixation was obtained at 45°C.

Picric Acid Mixtures. Bouin's fluid (p. 424) is a very useful fixative for Protozoa, as are many of its modifications, such as Allen's B_3 (p. 425) and that of Wetzel‡ who takes 3 parts of a concentrated solution of picric acid to 1 part of formol and 1 part of acetic acid. Brazil's§ alcoholic modification is as follows:

Picric acid	1.0 gm.
80 per cent alcohol	150.0 c.c.
Formol (full strength)	60.0 c.c.
Glacial acetic acid	15.0 c.c.

Picro-acetic is made up in various ways (p. 425). Dobell‖ added 1 part of glacial acetic acid to 3 parts of a saturated solution of picric acid in 90 per cent alcohol.

The writer finds Hollande's fluid useful, especially for certain flagellates. It is made up as follows:

* Maier, H. N. *Arch. f. Protistenk.*, 1903, ii, 73.
† Nowlin, N. *J. Parasitol.*, 1917, iii, 143.
‡ Wetzel, A. *Arch. f. Protistenk.*, 1925, li, 209.
§ Brazil, L. *Arch. de Zool. Expér. et gén.*, 1905, xxxiv, 69.
‖ Dobell, C. *Arch. f. Protistenk.*, 1914, xxxiv, 139.

Picric acid	4.0 gm.
Copper acetate	2.5 gm.
Formol	10.0 c.c.
Glacial acetic acid	1.5 c.c.
Distilled water	100.0 c.c.

Hollande washed pieces of tissue in water, but the writer finds 50 per cent alcohol satisfactory for washing smears after this fixative, as with the other picric acid fixatives just given.

Picro-mercuric Mixtures. Yocum* used the following:

Bichloride of mercury	2.0 gm.
Picric acid	1.0 gm.
Alcohol, 95 per cent	110.0 c.c.
Ether	20.0 c.c.
Glacial acetic acid	20.0 c.c.
Formol	50.0 c.c.

Another formula (Nowlin) is as follows:

Bichloride of mercury	1.0 gm.
Picric acid	1.0 gm.
Methyl alcohol	90.0 c.c.
Ether	10.0 c.c.
Glacial acetic acid	20.0 c.c.
Formol	50.0 c.c.
Distilled water to make total of	200.0 c.c.

Chromic Acid, Osmic Acid, and Other Mixtures. Chromo-acetic (p. 422), Champy's fluid (p. 423), Flemming's fluids (p. 423), Hermann's fluid (p. 423) and 1 per cent or 2 per cent osmic acid are all more or less used in the investigation of Protozoa. Some additional fixatives will be mentioned in the special section to follow.

c. Staining. The stains that one employs in any particular case need to be adapted (1) to the kind of animal to be stained, (2) to the fixative which has been used and (3) to the special purposes to be served. It is usually desirable to employ a number of different stains to gain a full knowledge of the structures of Protozoa.

Iron Alum Hematoxylin and Modifications. Heidenhain's method is fully described elsewhere (p. 469). It is probably the most generally useful single stain that may be employed for Protozoa, especially for the smaller forms and for sections of the larger ones. In general, the writer finds that the longer methods (twenty-four hours) give the best results, but briefer methods are often useful, e. g., the following:

Kornhauser's Quick Hematoxylin Method. After fixation and washing, place wet slides on an electric hot plate and add 4 per cent iron alum with a pipette. Keep the slide full (by additions from the pipette) and steaming for several minutes. Rinse in water, put back on the hot plate and add ripe 0.5 per cent hematoxylin with a pipette. Keep full

* Yocum, H. B. *Univ. of Cal. Pub. in Zool.*, 1918, xviii, 337.

and steaming for several minutes, then rinse in water and differentiate in cold 2 per cent iron alum under the microscope. When properly differentiated, wash thoroughly in running water, dehydrate, clear and mount in balsam.*

Regaud's Hematoxylin. Dissolve 1 gm. of hematoxylin in a mixture of 80 c.c. of distilled water, 10 c.c. of glycerin and 10 c.c. of absolute alcohol. Stain as with Heidenhain's after mordanting with 4 per cent or 5 per cent iron alum.

Kofoid and Swezy's Alcoholic Modification.† Mordant ten minutes in a 4 per cent solution of iron alum diluted with 10 parts of 50 per cent alcohol; rinse in 50 per cent alcohol; stain ten minutes to one hour in a 0.5 per cent solution of hematoxylin diluted with ten parts of 70 per cent alcohol. Differentiate in the iron alum; wash in 50 per cent alcohol or in water for two hours; dehydrate, clear and mount.

Dobell's alcoholic hematein modification.‡ From 70 per cent alcohol, place slides or smears in a 1 per cent solution of iron alum in 70 per cent alcohol for ten minutes or longer. Rinse again in 70 per cent alcohol and place in a 1 per cent solution of hematein in 70 per cent alcohol for ten minutes or longer. Rinse again in 70 per cent alcohol and differentiate in the alcoholic iron alum solution or in 0.6 per cent hydrochloric acid in 70 per cent alcohol. Wash thoroughly in 70 per cent alcohol, dehydrate, and mount.

Other hematoxylin stains frequently used for Protozoa are Delafield's hematoxylin (p. 468), Ehrlich's hematoxylin, Mallory's iron chloride hematoxylin (p. 469) and Mayer's hemalum (p. 469).

Counterstains are frequently used after hematoxylin stains. Among those more commonly employed are: eosin, erythrosin, orange G, methyl green, light green, acid fuchsin, Bordeaux red.

The Carmine Stains. Alum cochineal (p. 468), alum carmine (p. 467, borax carmine (p. 467), picro-carmine (p. 467), are also useful for staining Protozoa.

Polychrome Stains. Mallory's tricolor stain is frequently used, especially for sections. Sharp§ used the following modification. From water the preparation is treated thus:

	Seconds
(1) fuchsin S, 0.5 per cent aq. solution	forty-five
(2) distilled water	five
(3) phosphomolybdic acid, 1 per cent	sixty
(4) fresh distilled water	five
(5) aniline blue, orange G and oxalic acid	sixty
(6) distilled water	ten
(7) 95 per cent alcohol	one
(8) absolute alcohol	one
(9) carbol-xylol	one
(10) xylol to complete clearing, then mount.	

Giemsa's Stain, Wet Method. After fixation (e. g., in Schaudinn's fluid) wash and treat for ten minutes with a 0.5 per cent solution of sodium thiosulphate; wash in distilled water and stain several hours or overnight in Giemsa's stain (pp. 408, 447), diluting the stock stain 1 drop to 1 c.c. of distilled water. It is well to renew the stain after the first hour or two. The slide should be placed in the stain at an angle, with the smears or sections

* Kornhauser, S. I. *Stain Technology*, 1926, i, 78.

† Kofoid, C. A. and Swezy, O. *Proc. Am. Acad. Arts & Sc.*, 1915, li, 289.

‡ Dobell, C. *loc. cit.* p. 399.

§ Sharp, R. G. *Univ. Cal. Pub. in Zool.*, 1914, xiii, 43.

downward. Rinse in distilled water, then dehydrate and clear by the following acetone-xylol steps: (1) pure acetone, 95 parts, xylol, 5 parts; (2) acetone, 70 parts, xylol, 30 parts; (3) acetone, 30 parts, xylol, 70 parts; (4) pure xylol; then mount in neutral balsam.*

Short Giemsa Method (p. 408). Fix wet smears in equal parts of methyl alcohol and ether; transfer to Giemsa's stain, 1 drop of stock stain to 15 c.c. of distilled water for eight to ten minutes; wash in distilled water till pink color appears; dry in air and mount immediately in balsam.

Mann's Methyl Blue-eosin Method. The stain is prepared by mixing together:

1 per cent methyl blue in distilled water	35 c.c.
1 per cent eosin in distilled water	45 c.c.
Distilled water	100 c.c.

Arndt† modifies this by taking 3 parts of distilled water and adding 1 part of the 1 per cent methyl blue and 1 part of the 1 per cent eosin. (Note: *methyl blue* is used, not methylene blue.)

Shorter method of staining: transfer sections or smears from water to the stain for five to ten minutes, depending on the special needs, the thickness of the sections or smears, and the fixative used. Rinse in water, dehydrate, clear and mount.

The longer method, as employed by Dobell.‡ Stain four to twelve hours, wash in distilled water, then differentiate in a solution made by adding a few drops of saturated orange G to 100 c.c. of 70 per cent alcohol. Wash in water, dehydrate with graded alcohols, clear and mount.

Alcoholic Eosin-methylene Blue Method.§ From absolute alcohol place in 1 per cent alcoholic eosin for one minute; wash in water; stain for one minute in 1 per cent methylene blue in distilled water; wash in water; dry slide with a cloth or blotter, but leave tissue moist, differentiate quickly in absolute alcohol; clear in xylol or benzol and mount in balsam.

Methods for mitochondria in the Protozoa are the same as the methods used for Metazoa (p. 198), or the following method of Causey|| may be employed. Fix one to two hours in 1 per cent osmic acid or in osmic vapor and stain as follows: mordant in 5 per cent iron alum for thirty to forty minutes; rinse in distilled water, stain in Regaud's hematoxylin (p. 202) thirty to forty minutes; wash in water and destain in the 5 per cent iron alum, watching under the microscope. The time for destaining will vary with the size and species.

Methods for Golgi Bodies. King and Gatenby¶ demonstrated Golgi bodies in Opalina by the following method: Fix the host's rectum for twenty-four hours in Champy's fluid (p. 201); wash under the tap for twenty-four hours, then cut into small pieces and keep in 2 per cent osmic acid for four days at a temperature varying from 30° to 60°C. Wash the pieces in distilled water, dehydrate, imbed, and make thin sections. Mount the sections unstained. (For the method of Nassonov, see p. 414.)

d. Sectioning. The general principles for sectioning Protozoa are the same as those for sectioning tissues of the Metazoa (see Part 1). When Protozoa occur in the tissues of host animals, these tissues are handled just as other tissues are. Free-living Protozoa are usually sectioned en masse, i. e., a large number concentrated together, or as single individuals

* Wenyon, C. M. Protozoology. Lond., 1926.

† Arndt, A. *Arch. f. Protistenk.*, 1924, xlix, 1.

‡ Dobell, C. The Amoebae living in Man. Lond., 1919.

§ Lim, R. K. S. *Quart. J. Micr. Sc.*, 1919, lxiii, 541.

|| Causey, D. *Loc. cit.* p. 396.

¶ King, S. D. and Gatenby, J. Brontë. *Quart. J. Micr. Sc.*, 1926, lxx, 217.

Sectioning en masse. The chief problem here is that of concentration. For this purpose, all steps up to clearing may be done by sedimentation or with the centrifuge. Clearing may be done with xylol or with chloroform for paraffin imbedding. There are a number of methods of handling.

(1) Transfer the concentrated group of Protozoa with a pipette from xylol to a paper boat made by folding heavy paper into a small rectangular open box. Lower this box or boat into melted paraffin. Several changes of paraffin are advisable to insure the removal of all the xylol.

(2) Make a mold in hard paraffin with a brass rod which is square in cross section (Sharp).* Into this mold run the melted paraffin containing the animals and allow to cool. The hard paraffin of the mold may be tinted by sudan III to differentiate it better from the imbedding paraffin.

(3) Lefevre's watch glass may be used for imbedding. This has a straight-sided groove at the bottom. Pour the melted paraffin containing the Protozoa into the watch glass and sweep the organisms into the groove with a small brush or other agent. After hardening, trim off the projecting ridge of paraffin from the groove and section.

(4) Calkins† method. Fix ciliates in a test tube and add zooglea from an old Paramecium culture. Shake the tube to mingle the ciliates with the zooglea and again after each change of fluid through washing and clearing. There is thus obtained a well-matted mass which is stained in eosin to facilitate observation. Imbed in the same manner that a piece of tissue would be imbedded.

Sectioning Individuals. Single individuals may be handled in a watch crystal or round bottomed vial under the dissecting microscope. Staining with eosin helps in the recognition of the animal. The individual may be transferred from dish to dish with a pipette or may be left in one dish and the fluids changed with a pipette.

Imbed in the small white porcelain dishes commonly used for water colors. From xylol in the vial or watch crystal, transfer the object to xylol in the dish. Replace xylol by melted paraffin which is prevented from hardening by gently heating the stage of the microscope with a small lamp. Make several changes of paraffin. In the final paraffin, orient the object in the center of the dish with a fine glass needle with a bead tip. The dish is set in ice water to a depth that reaches to the top of the dish without overflowing it. As the paraffin cools from the bottom up, additional melted paraffin is added to prevent the depression that usually forms during cooling.‡

Collodion imbedding is sometimes used (see Part 1) and is described by Scott§ for sectioning Balantidium.

Combined collodion and paraffin imbedding may be accomplished as follows: From alcohol transfer to alcohol-ether, then to ether, using the centrifuge and decanting the supernatant fluids. To the organisms at the

* Sharp, R. G. *Loc. cit.* p. 401.

† Calkins, G. N. *J. Exper. Zool.*, 1919, xxvii, 293.

‡ Fry, H. J. *Anat. Record*, 1927, xxxiv, 235.

§ Scott, M. J. *J. Morphol. & Physiol.*, 1927, xlix, 417.

bottom of the tube, add a drop or two of liquid collodion. When evaporation has reduced this to the consistency of a thick syrup, the lenticular drop is taken out and placed in alcohol-chloroform, then pure chloroform, then imbedded in paraffin.*

2. Special Section. *a. Intestinal Protozoa. Fresh Material.* (For examination of living animals see pp. 389–395.) For examination of fresh material, dilute the intestinal contents or feces on a slip with a drop of saline solution and then treat with a 0.1 per cent to 0.5 per cent solution of eosin or with dilute iodine solution, or both together.† The eosin will stain nearly all the material in the preparation except the living Protozoa and their cysts, thus bringing them into relief against the pink background. The iodine solution (Lugol's solution diluted about 1 to 10 with water) will kill and stain the animals as well as stain the other objects, but will bring out the nuclei, the glycogen bodies in some of the protozoan cysts, and the motile organs of the vegetative ciliates and flagellates. Of these two methods, the iodine treatment is probably the more useful.

Smears of intestinal protozoa for fixation and staining may be made either on slips or cover glasses. An effort is made to get a thin, even film which is not so fluid that it will wash off on fixation, and not so viscid that it will dry before it can be fixed. The best results can be secured only after some experience. In most cases there is enough coagulable material in feces or intestinal contents to cause it to adhere readily to the slip or cover upon fixation. Dilution with weak salt solution (p. 393) is usually required, but occasionally such material is so fluid or so lacking in coagulable material that egg albumen will need to be added. The writer finds a small, curve-pointed forceps a convenient tool for making smears, and usually makes them on cover glasses, passing them through the reagents in Petri dishes, 8 to 12 in a dish. Feces or intestinal contents may also be fixed in bulk and for the large ciliates of ruminants this is usually advisable. Such material may be handled in one or another of the methods described in the general section (pp. 396–397).

Fixation. Choice of methods depends on the animals and the special purposes to be accomplished. Hot Schaudinn's fluid is most commonly employed but should always be supplemented by other fixatives. Zenker's fluid is sometimes used for subsequent staining with Mallory's triple stain or wet Giemsa stain. Chromic or osmic mixtures are useful for bringing out special structures, such as parabasal bodies (p. 399), mitochondria (p. 402) Golgi bodies (p. 402) etc.

Staining. Heidenhain's hematoxylin is the most generally useful stain except for the larger ciliates which should be stained with hemalum, Delafield's hematoxylin and similar stains. In both cases, appropriate

* Collin, B. *Arch. de Zool. expér. et. gén.*, 1912, li, 1.

† Donaldson, R. *Lancet*, 1917, p. 571.

counterstains may be used. More detailed methods for the ciliates are given further on (p. 412).

Washing Cysts. Mix thoroughly 5 to 10 gm. of feces in 100 to 200 c.c. of distilled water, sterile tap water or weak salt solution, then strain through several thicknesses of cheese cloth to remove coarser particles, and place the suspension in a tall vessel. The cysts and other heavier materials will fall to the bottom in two hours or more, when the supernatant fluid is poured off and fresh fluid is poured on and thoroughly stirred up. Allow to settle again, pour off the supernatant liquid, and pour on fresh fluid. Continue the process until the supernatant liquid is clear. The process may be hastened by using smaller quantities in a centrifuge. This method does not kill the cysts.

Boeck's Concentration Method. Place 1 gm. of stool in 30 c.c. of normal salt solution and thoroughly stir with a Hamilton-Beach "Cyclone" electric mixer for ten minutes. While still stirring, add 5 c.c. of ether. Cease stirring when the emulsion begins to foam the second time (i. e., 2 to 3 minutes longer). Quickly transfer to a separatory funnel and allow to stand for five to seven minutes. Draw off 15 c.c. of fluid at the bottom of the funnel and centrifuge for three minutes at 1600 revolutions per minute. Draw off the supernatant liquid and examine the residue.* The stirring may be done with a rod or woven-wire ladle held in the hand, but requires a much longer time.

De Rivas' Concentration Method. Place 1 or 2 gm. of feces in a test tube and add 5 to 10 c.c. of 5 per cent acetic acid. Closing the mouth of the tube with a rubber stopper or with the thumb covered by a sheet of rubber, shake the mixture vigorously till a more or less homogeneous suspension is produced. When feces are especially hard add a few glass beads to the material in the tube or break up the lumps with an applicator. Allow the suspension to stand for one-half to one minute during which time the acid appears to act on the fecal material, better preparing it for further treatment. It is well to filter the suspension through a double thickness of cheese cloth or through a finely woven copper wire mesh which can afterwards be cleaned by burning over a bunsen flame. Place about 5 c.c. of the suspension in an ordinary centrifuge tube and add an equal amount of ether. Close the tube and shake vigorously with the hand, holding the tube in a horizontal position. Next centrifuge for a few minutes. The material in the tube separates into four layers, the etherial extract at the top, the detritus plug near the middle, the acetic acid solution below and the sediment at the bottom. This sediment will contain the cysts of Protozoa and the eggs of worms, yeasts, and similar bodies.† This is a very efficient concentration method.

* Boeck, W. C. *Univ. Cal. Pub. in Zool.*, 1917, xviii, 145.

† De Rivas, D. *Am. J. Trop. Med.*, 1928, viii, 63.

Intestinal amebae are best fixed in Schaudinn's fluid or other sublimate mixtures. An exception is Dientamoeba fragilis which, the writer's experience, stains better after Bouin's or other picric acid mixture. The writer has also found that picromercuric will produce more "budding" on the cysts of certain amebae than will Schaudinn's. The most useful stain for both vegetative stages and cysts is Heidenhain's hematoxylin, with or without counterstains.

Intestinal Flagellates. Fix in Schaudinn's, Bouin's, Hollande's and picromercuric mixtures for ordinary work. The writer has found the last two especially good for Giardia. For special structures like the parabasal body in Trichomonads, use chromic acid or chrom-osmic mixtures without acetic acid. Stain with Heidenhain's hematoxylin and counterstain with Bordeaux red, eosin, or light green and acid fuchsin. For staining the flagella see pp. 411, 412.

Intestinal Ciliates. In most cases smears may be made in the usual way but the material from ruminant stomachs does not smear well unless albumen is added, and this leads to some distortion. For these ciliates, fixation in bulk in hot fixatives is recommended. These can then be treated as indicated in the general section (p. 397). Fix with the sublimate, picric acid, or chrom-osmic mixtures, and stain small forms with Heidenhain's hematoxylin followed by Bordeaux red, eosin, or other counterstain. Stain the larger ciliates with hemalum, Delafield's hematoxylin or similar stains and counterstain as with Heidenhain's. While Heidenhain's does not usually do so well with larger forms, Sharp reported good results with a special modification (p. 413).

For finer details section the larger ciliates (pp. 403, 413). Where the host is small, e. g., a frog, the intestine may be fixed and sectioned. In the case of Ruminants, fix the ciliates en masse and then handle by methods previously given (p. 403). Scott preferred imbedding Balantidium in collodion to paraffin for sectioning. Where the ciliates invade the tissues of the host (Balantidium coli) fix and section the tissues before staining.

Intestinal Sporozoa: Gregarinida. Gregarines are limited to invertebrate hosts but are not confined to the digestive tract of the hosts. However, since a majority do occur in the digestive tract, and all may be treated by the same general technique, they will be considered here as a unified taxonomic group. While gregarines are coelozoic through most of their development, they are frequently intracellular in the earlier stages, and even in later stages may remain attached to the host's tissues by special hold-fasts. Hence to get all stages and to see the relation of the parasites to the host's tissues, it is well to fix the parasitized organs and section them. Smears may be made in the usual way and are necessary for study of the entire animals.

Fix smears with sublimate or picric acid mixtures and stain with Delafield's or Ehrlich's hematoxylin followed by eosin or other counterstain.* Berlin stained thin sections of monocysted gregarines with Hansen's hematoxylin, picro-fuchsin, Giemsa's stain and Heidenhain's hematoxylin, with counterstains of Bordeaux red, orange G and safranin plus light green.

Intestinal Sporozoa: Coccidia. These are intracellular parasites but occur most commonly in the intestinal epithelium of their hosts and the oocysts are discharged into the lumen of the intestine to mingle with its contents and the feces. In many cases, the oocysts are highly resistant to chemical action and cannot be fixed and stained as can the other stages. In these cases developmental stages within the oocyst must be watched in the living condition. This may readily be done if the oocysts are kept moist. For the intracellular stages make smears, fix and stain like fecal smears, or fix the host's tissues, cut into pieces of convenient size, and section. These two methods are also advised for organs other than the digestive tract. Dobell† recommends Schaudinn's, Bouin's and Flemming's fixatives for smears, and Bouin's picro-acetic (p. 424) and sublimate-formol (3 parts of sat. aq. sol. of sublimate to 1 part of full strength formol) for tissues for sectioning. He cautions against allowing sea water to come into contact with the tissues of the marine hosts with which he worked. For staining smears, Dobell recommends Delafield's hematoxylin, hemalum, picrocarmine, borax carmine, alum carmine and safranin (after Flemming's fixative). For sections he prefers his alcoholic hematein (p. 401), Heidenhain's, Mallory's iron chloride hematoxylin (p. 469) and others with counterstains of eosin, orange G, light green and Bordeaux red.

b. Blood-inhabiting Protozoa. (For methods of obtaining blood and of making blood smears see p. 244.)

Thin films or smears are made in the usual way on slips or covers, an attempt being made to get an even film, so thin that the corpuscles will lie side by side but not overlie each other. The films are either dried at once, fixed before drying, or fixed in the cytological fixatives without drying.

Fixation. Blood stains made up in methyl alcohol act both as a fixative and stain. Aqueous stains require fixation before staining. The fixatives most generally employed are ethyl alcohol (95 per cent to 100 per cent), pure methyl alcohol, or a mixture of equal parts of alcohol and ether. Fix for five to thirty minutes, depending on the material and stain to be used. Fixation before drying with osmic acid vapor for twenty to thirty seconds, or with formaldehyde vapor for one to two minutes is preferred by some, or the vapor fixation may be followed by alcohol before drying. (For fixation without drying see p. 248.)

Staining. Before staining it is well to mark off the smear area on a slip with a wax pencil to prevent the stain from spreading over the entire

* Watson, M. E. *Univ. Ill. Monographs*, 1916, ii, 211.

† Dobell, C. *Parasitology*, 1925, xvii, 1.

surface. The aqueous stains which require previous fixation are Giemsa's, borax-methylene blue, and methylene blue-eosin.

Giemsa's Stain. (For preparation of the stock stain, see p. 246.) For staining, dilute the stock stain by adding 1 drop to each cubic centimeter of distilled water and add to the previously fixed smears. To avoid precipitates forming on the blood film, float cover smears on the surface of the stain or place the slide smears in a dish of the stain with the film side down. For the latter purpose a Petri dish may be used, the slip being supported at one end. Stain for ten to thirty minutes, depending on the material; wash thoroughly in distilled water; dry and mount in neutral balsam or leave unmounted.

Borax-methylene Blue (Manson). Dissolve 2 gm. of methylene blue in a boiling solution of 5 gm. of borax in 100 c.c. of distilled water. Upon cooling, this stock is ready for use. De Rivas* dissolves 2 gm. of methylene blue in 10 c.c. of 95 per cent alcohol, then adds 100 c.c. of water and 5 gm. of borax. After shaking well he allows the mixture to stand over-night and then filters. For staining previously fixed smears, add 1 to 2 c.c. of the stock stain to 100 c.c. of distilled water. Stain about one minute, rinse thoroughly in water, dry and mount.

Methylene blue-eosin is prepared as follows:

Methylene blue, concentrated aq. solution	60 c.c.
Eosin, 0.5 per cent in 75 per cent alcohol	20 c.c.
Distilled water	20 c.c.
Potassium hydrate (20 per cent)	12 drops

Stain five to ten minutes, dry, and mount in balsam.

Wright's Blood Stain. (For preparation, see p. 460.) Previous fixation is not required. Cover the blood film with the stock stain which is allowed to act as a fixative for one minute, then add distilled water, drop by drop, until a green scum begins to form on the surface. The amount of water added is usually about one-half the amount of stain, or not more than an equal amount. After dilution allow the stain to act for two to three minutes, then wash off thoroughly with distilled water, dry the film in the air and mount.

Leishman's stain may be purchased as a dry powder which is dissolved in pure methyl alcohol in the proportion of 0.15 gm. to 100 c.c. Allow this stain to act on a smear as a fixative for one-half to one minute, then dilute by two volumes of distilled water and allow to stain for two to five minutes longer. Wash off with distilled water, dry and mount.

Jenner's stain is made up as follows: Mix 100 c.c. of a 1 per cent aqueous solution of eosin (Grübler's) with 100 c.c. of a 1 per cent aqueous solution of methylene blue (Grübler's). Allow mixture to stand twenty-four hours and filter. Dry the filtrate at 65°c., wash in water, again dry, and powder it thoroughly. Dissolve the powder in pure methyl alcohol in the proportion of 0.5 gm. to 100 c.c. Stain smears two to five minutes, wash in water, dry and mount.

Russell's Combination Stain. Place the blood smear (on a slip) for two minutes in undiluted Wright's stain (or other methyl alcohol blood stain); without washing place in a dish of tap water for three to five minutes; without washing transfer to dilute borax-methylene blue (1 c.c. of stock stain to 100 c.c. of water, made up fresh) for twenty to

*De Rivas, D. Human Parasitology, Phila., 1920.

forty seconds. Wash quickly in tap water, dry and mount. This method combines the nuclear staining properties of Wright's stain with the cytoplasmic staining properties of borax-methylene blue.*

Thick films are often made for diagnoses, when parasites are suspected but are few in number, and dehemoglobinized before staining. Take a large drop of blood and spread it on a slip over an area 16 to 18 mm. across, or make a series of smaller drops on a slip without much spreading and allow to dry thoroughly. Gently move the slip about in a glass containing a 2 per cent solution of formalin to which has been added 1 per cent of glacial acetic acid. After the color has disappeared, treat the slip in the same way in a glass of water to remove all traces of acid. Wash gently in distilled water and stain with dilute Giemsa's stain (1 drop to 1 c.c. of distilled water) for twenty to thirty minutes, wash in distilled water and dry without heat or blotting paper (Stitt†). Other stains can, of course, be used.

Wet Films. Fix blood smears with the usual fixatives, (Schaudinn's etc.) then wash and stain either with polychrome stains (e. g., wet Giemsa p. 401) or with hematoxylin stains, with eosin or other counterstains. Such preparations are thought to be better for cytological details.

Relief Stains. Instead of staining the parasites, the background may be dyed, leaving the parasites clear on a colored background. This method is more especially useful for the plasma-inhabiting forms, such as the trypanosomes. (1) Mix a drop of fresh blood on a slip with a drop of a thick suspension of India ink and make a smear of this in the usual way, then dry and mount in balsam or examine with immersion oil without a cover glass. (2) Employ in the same way a saturated solution of opal blue and similar stains (Bresslau‡) or nigrosin (Coles§) or a mixture of 3 parts of a saturated solution of china blue to 1 part of a saturated solution of cyanosin which has been allowed to stand one day after sterilization by boiling.‖

Hemoflagellates. The methods given in the preceding paragraphs are applicable not only to flagellates found in the blood of their hosts, but also to related flagellates (Crithidia, Herpetomonas, Cryptobia), found in the digestive tracts of invertebrate hosts and to tissue-inhabiting hemoflagellates such as Leishmania. In these cases either make dried smears and stain like blood films, or fix with the usual fixatives, and stain with cytological stains. The latter choice is usually made for the study of cytological details. Tissues may be sectioned and the sections stained by the usual methods. One additional method may be mentioned.

Gold chloride method for myonemes of trypanosomes. Fix wet smears in Schaudinn's fluid. After washing, leave for twenty-four hours in 1 per cent gold chloride. Wash in water, then place in 1 per cent formic acid and set in

* Russell, F. F. *J. Am. Med. Ass.*, 1915, lxiv, 2131.

† Stitt, E. R. Practical Bacteriology, Blood Work and Animal Parasitology. Ed. 7, Phila., 1923.

‡ Bresslau, E. *Arch. f. Protistenk.* 1921, xliii, 467.

§ Coles, A. C. *Watson's Microscope Rec.*, 1927, p. 23.

‖ Eisenberg, *Zentralbl. Bact., Ref.*, 1912, liv, 145.

strong light until a purple-red color appears. If direct sunlight is available, two to three hours should suffice. Dehydrate in graded alcohols, clear in xylol and mount in cedar oil.*

Hemosporidia. In addition to malarial organisms, other blood-inhabiting Sporozoa, including hemogragarines and piroplasms may be handled by the methods previously given. In most cases it is desirable to supplement dried film methods by wet fixation and cytological staining.

c. Sarcodina. Free-living Sarcodina (for endozoic amebae, see p. 393) are so different among themselves in size and organization that it is difficult to formulate general rules for their treatment. For cytological details they are usually fixed with sublimate, picric acid or chrom-osmic mixtures followed by staining of the smaller species entire with Heidenhain's, Delafield's hematoxylin, or hemalum, with counterstains, or with wet Giemsa's stain, Mallory's triple stain or Mann's methyl blue-eosin. Larger kinds may be stained entire with hemalum or Delafield's hematoxylin or sectioned and stained with Heidenhain's hematoxylin or the other stains just given.

Amebae. Dobell† (p. 407) prefers staining with his alcoholic hematein (p. 401) after picric acid fixatives. Wiener‡ proceeds as follows:

> Spread culture amebae on a slide and allow them to dry. Fix with alcohol, then place for five minutes in 1 per cent iodine; rinse in water and let drain. Stain one and one-half minutes in Loeffler's methylene blue (p. 92); rinse in water and drain, then one minute in a concentrated solution of eosin diluted with 3 volumes of water. Rinse the slide and mount in balsam.

Foraminifera. Separate living Foraminifera from sand and debris by stirring up the collection mass in water and allowing it to settle for a brief time. The organisms will settle to the bottom of the container with other heavier material, but the finer particles will remain suspended and can be decanted off (Carpenter).§

Dead Shells. Carefully dry the collection mass in an oven, stirring several times, then sift into water. The air-filled shells should float on the surface of the water and the sand go to the bottom (Carpenter). Or, thoroughly stir the collection mass into a vessel of water until everything that will sink has gone to the bottom. Place a teaspoonful at a time of the wet material in a saucer or similar dish, spread out and cover with water to a depth of about 5 mm. Rotate the dish carefully and the Foraminifera shells will come to the surface of the sand and can be removed with a ball pipette (Vorce).‖

Thin sections of Foraminifera shells for the study of the finer details are prepared by grinding. Usually the shells are attached to a glass slip

* Ogawa, M. *Arch. f. Protistenk.*, 1913, xxix, 248.

† Dobell, C. *Loc. cit.* p. 407.

‡ Wiener, E. *Arch. f. Protistenk.*, 1918, xxxix, 105.

§ Carpenter, W. B. The Microscope and its Revelations. Ed. 8, Lond., 1901.

‖ Vorce, C. M. *Am. Month. Micr. J.* 1880, i, 24.

with hard balsam and ground on appropriate grinders much as teeth are ground (p. 265).

For cytological details prepare by fixing with the usual fixatives, decalcify with weak hydrochloric acid in 60 per cent alcohol* and stain entire with borax carmine, hemalum, Delafield's, etc., or section and stain with Heidenhain's with counterstains.

Heliozoa. Fix with the usual fixatives and stain smaller forms entire with Heidenhain's or Delafield's hematoxylin, carmalum, borax carmine, or alum carmine.† Stain larger forms like *Actinosphaerium* entire with hemalum and similar stains or section and stain with Heidenhain's hematoxylin with counterstains. Axopodia are best demonstrated by silver impregnation methods‡ (p. 344).

Radiolaria. Separate skeletons from recent deposits by washing with a gentle stream of water through sieves of various grades. The smallest skeletons will go through all the sieves, but will fall to the bottom of the receptacle and the finer suspended particles of dirt, etc., can be decanted off.§ If Foraminifera shells are to be removed also, treat with dilute nitric acid.

Fossilized deposits require repeated treatment with hot soda solutions alternated with drying. This treatment may destroy the more delicate skeletons (Earland).

For cytological details, fix skeletonless Radiolaria, or those with loose spicules, with sublimate mixtures, Flemming's or Hermann's fluids,‖ then section and stain with Heidenhain's or Kleinenberg's hematoxylin, etc.¶ Stain animals with skeletons entire with hemalum or the carmine stains.

d. Mastigophora. Animal flagellates can be treated for the most part by the methods described for the intestinal species (p. 404). Hemoflagellates are treated in a separate section (p. 409). One of the difficulties in the handling of flagellates is to get a good flagellar stain. For many forms the flagella can be demonstrated by making dried smears and staining as for blood flagellates or by using the relief staining method (pp. 409, 413). Such preparations do not, as a rule, give good results for the remainder of the cell. Two methods of staining flagella are here offered:

Löffler's Stain for Flagella. To 10 c.c. of a 20 per cent solution of tannin add 5 c.c. of a cold saturated solution of ferrous sulphate and 1 c.c. of a solution of (either aqueous or alcoholic) fuchsin, methyl violet or "wollswarz." Fix cover glass preparations by heat (do not over-heat). While still warm, treat with the above mixture as a mordant, heating

* Schaudinn, F. *Ztschr. f. wiss. Zool.*, 1895, lix, 191.

† Zuelzer, M. *Arch. f. Protistenk.*, 1909, xvii, 135.
Belar, K. *Arch. f. Protistenk*, 1923, xlvi, 1.

‡ Rumjantzew, A. & Wermel, E. *Arch. f. Protistenk*, 1925, lii, 217.

§ Earland, A. *Nature*, 1922, p. 110.

‖ Huth, W. *Arch. f. Protistenk.*, 1913, xxx, 1.

¶ Bogert, A. I. *Teil. Zool. Jahrb. Abt. f. Anat. u. Ontog.*, 1900, xiv, 203.

for half a minute until liquid begins to vaporize. Wash in distilled water, then in alcohol, then treat, as with the mordant, with the stain which consists of a saturated solution of fuchsin in aniline water, neutralized by a little 0.1 per cent soda solution. This is one of the tannin methods of which there are many variants.

Hollande's Method for Demonstrating Flagella.* Fix tissues in the picro-formol-copper-acetate-acetic mixture already given (p. 400) for three to five days and wash in water for twenty-four to forty-eight hours. Sections (or smears) are then stained with Heidenhain's in the usual way, using 3 per cent iron alum as a mordant. After the usual differentiation, place in 1 per cent aqueous eosin for two to twenty-four hours; wash several seconds in distilled water to remove the excess of eosin; place in a 1 per cent aqueous solution of phosphomolybdic acid for five to ten minutes; wash with tap water thirty seconds; place in 0.2 per cent to 0.5 per cent aqueous solution of light green for several seconds to one minute, according to the time in the eosin and phosphomolybdic acid; dehydrate rapidly in graded alcohols; differentiate in 96 per cent alcohol for one to ten minutes, examining from time to time under the microscope. Arrest the differentiation by placing in pure amyl alcohol in which dehydration is completed. A second bath in amyl alcohol is advisable. Clear by passing first into equal parts of amyl alcohol and xylol, then into xylol, and mount.

Chrysomonadina. Fix in a mixture of 2 volumes of a saturated aqueous solution of boric acid and 3 volumes of a saturated solution of mercuric chloride for three hours and wash in water† or fix with osmic vapor or Schaudinn's fluid,‡ and stain with Bordeaux red and Heidenhain's or wet Giesma, or Mallory's triple stain.

Volvocidae. Fix with Schaudinn's or Flemming's and stain with Heidenhain's with counterstains or with safranine and light green.§

Euglenoids. For flagellum and gullet fix one to three hours in Altmann's fixative (p. 199) and stain with Regaud's hematoxylin (p. 401). For blepharoplasts, cytoplasmic rhizoplasts and nucleus, fix in hot Schaudinn's fluid two to ten minutes and stain with Heidenhain's (twenty-four hours) preceded by immersion for twenty-four to forty-eight hours in a 0.2 per cent solution of Bordeaux red.‖ These methods are very useful for other flagellates.

Dinoflagellates. Fix in Flemming's fluid. Stain entire organisms with Delafield's or picrocarmine, sections with Heidenhain's¶ or fix with Schaudinn's and stain with Bordeaux red-Heidenhain's or borax carmine or eosin and methylene blue.**

e. Infusoria. The choice of technique will depend on the nature of the material and the special results desired. For most purposes, the usual fixatives, Schaudinn's, Bouin's, Flemming's, etc., are adequate. For smaller ciliates, Heidenhain's hematoxylin, with appropriate counterstains such as eosin, light green, or especially prestaining with Bordeaux red will give an adequate picture of the structural organization. Heidenhain's is not so well adapted to the larger ciliates, although Sharp†† and others have claimed success with the following modification:

* Hollande, A. C. *Arch. de Zool. expér. et. gén.*, 1920, lix, 75.
† Zacharias, O. *Zool. Anz.*, 1899, xxii, 70.
‡ Doflein, F. *Arch. f. Protistenk*, 1922, xliv, 149.
§ Hartmann, M. *Arch. f. Protistenk.*, 1918, xxxix, 1.
‖ Hall, R. P. and Powell, W. N. *Trans. Am. Micr. Soc.*, 1926, xlv, 256.
¶ Lauterborn, R. *Ztschr. f. wiss. Zool.*, 1895, lix, 167.
** Hall, R. P. *Univ. Cal. Pub. in Zool.*, 1925, xxviii, 29.
†† Sharp, R. G. *Loc. cit.* p. 401.

Sharp's Modification of Heidenbain's for Larger Ciliates. Mordant twenty-four to thirty-six hours in a 1 per cent solution of iron alum; wash with distilled water; stain twenty-four hours with 0.3 per cent aqueous hematoxylin; wash in tap water and differentiate in the 1 per cent iron alum. Wash, dehydrate, etc., as usual.

For whole mounts of larger ciliates, stain with hemalum, Delafield's hematoxylin, or similar stains, counterstain with eosin, light green, orange G or Bordeaux red.

Sections of ciliates are necessary for finer details. Stain thin sections, 2 μ to 4 μ thick, in Heidenhain's hematoxylin with a counterstain, or Mallory's triple stain (see Sharp's modification p. 401) will give good results.

Relief Staining. Surface markings, ciliary rows, etc., are seen in sections, but whole mounts may be prepared by the methods of Bresslau, Coles and Klein. Among the stains used are (1) a mixture of 3 parts of a saturated solution of china blue with 1 part of a saturated solution of cyanosin (Bresslau's "cyanochin"), (2) a mixture made by adding 4 to 6 drops of a 6.5 per cent aqueous solution of phlorenrhodamin to 1 c.c. of a 10 per cent aqueous solution of opal blue (Bresslau, p. 409), (3) a saturated aqueous solution of nigrosin (Coles, p. 409). Place a drop of water or culture containing the ciliates in the middle of a slip and near it a drop of the stain. Thoroughly mix the two, spread in a thin layer, and allow to dry in the air. Mount in balsam or examine without a cover. The surface depressions of the animals retain the stain thus outlining the positions of the cilia.

*Silver Impregnation Method.** Spread a drop of culture containing the ciliates in a thin layer on a slip or cover glass and allow it to dry in the air. Place for six to eight minutes in a 2 per cent solution of silver nitrate in distilled water, rinse well in distilled water contained in a white porcelain (or other) dish and set in strong daylight. Reduction follows in from four to ten hours, according to the intensity of the light. When the silver nitrate has become sufficiently reduced (test by examination with the microscope), rinse well, dry and mount in balsam. A network of "silver lines" becomes demonstrated by reducing the silver nitrate.

Sharp† studied the surface markings of Diplodinium by fixing in 4 per cent formalin and mounting unstained in styrax.

Cilia. For fresh-killed animals, fix with osmic acid or osmic vapor and then treat with 5 per cent soda solution (Maier‡), or with bromine vapor.§ The finer structures can best be studied in thin sections. Fix in Schaudinn's or Flemming's (Maier) or bibromate-acetic (potassium bibromate, 3 parts; glacial acetic acid, 5 parts; water, 100 parts), (Wetzel, p. 399), or fix in the regular fixatives followed by treatment with brome-bromine water (1 drop of pure bromine in 2 to 3 c.c. of strong bromine water) for ten to twelve hours, then wash with distilled water.§ Section and stain with Bordeaux red-Heidenhain, Mallory's triple, or other combination staining methods.

Trichocysts. Stain with Heidenhain's, especially in sections, after strong fixatives such as Schaudinn's fluid. To cause trichocysts to discharge in fresh preparations use weak concentrations of such acids as acetic, tannic, picric, osmic, chromic, etc., or bromine.

Trichites. Prepare these pharyngeal rods for study by the methods given above for cilia. MacDougall‖ found that they were not affected by dilute acids or dilute alkalies but were digested by artificial gastric juice (0.2 per cent HCl and 1 per cent pepsin in 100 c.c. of water), which indicated a protein composition.

* Klein, B. M. *Zool. Anz.*, 1926, lxvii, 160.
† Sharp, R. G. *Loc. cit.* p. 401.
‡ Maier, H. N. *Loc. cit.* p. 399.
§ Khainsky, A. *Arch. f. Protistenk*, 1910, xxi, 1.
‖ MacDougall, M. S. *Quart. J. Micr. Sc.*, 1925, lxix, 361.

Contractile Vacuoles. Stain in a fresh condition with alizarin blue (Grübler's) (Howland, p. 394). They show well in sections prepared by the methods for demonstrating Golgi bodies. Fix with Altmann's fixative (p. 199) and stain with Heidenhain's hematoxylin,* or use the method of Nassonov.† Fix for twenty-four hours in either of the following:

(1) Potassium bichromate, 3 per cent..... 2 parts
Chromic acid, 1 per cent..... 2 parts
Osmic acid, 2 per cent..... 1 part

(2) Potassium bichromate, 6 per cent..... 1 part
Chromic acid, 1 per cent..... 1 part
Osmic acid, 2 per cent..... 1 part

Wash thoroughly in distilled water and place in 2 per cent osmic acid for three to four days at a temperature of 35°c. Wash thoroughly in water, and dehydrate in graded alcohols. Small species can be mounted whole, but larger ones, e. g., Paramecium, need to be sectioned (paraffin). Subsequent staining is unnecessary. From the selective action of the contractile vacuole apparatus on osmic acid, Nassonov concluded that it is homologous with the Golgi apparatus of Metazoa.

Neuromotor Apparatus. Fix in Schaudinn's, Zenker's, Flemming's, etc. Stain in Heidenhain's or Mallory's triple (see Sharp's modification, p. 401). These structures are usually stained well by Heidenhain's hematoxylin in thin sections. The writer finds that the system of fibers by Rees‡ can be demonstrated in Paramecium by fixation with Schaudinn's fluid at 75°c., and staining entire with hemalum or Delafield's hematoxylin and mounting whole.

f. Sporozoa. Myxosporidia. (Other sporozoa are considered elsewhere: Gregarinida, Coccidia p. 407; Haemosporidia, p. 410). Myxosporidia are either histozoic or celozoic. For those in tissues either smear on slips or covers, then fix and stain like intestinal smears (p. 404), or fix the containing tissues, imbed and section. Stain the sections with the hematoxylin or polychrome stains. Remove coelozoic forms from the host and make smears of them, or fix the containing organs and section.

Fix with Schaudinn's, Bouin's, Flemming's and Hermann's fluids, or Worcester's fluid.§ Stain with Heidenhain's or Delafield's with or without counterstains, or with Mallory's triple, or wet Giemsa, for both smears and sections.‖

Shrinkage of spores. Kudo¶ tried a number of common fixatives and found that they caused a decrease of about 14 per cent in the sutural diameter of the mature spores of Leptotheca ohlmacheri. In making measure-

* Young, R. A. *Science*, 1924, lx, 244.
† Nassonov, D. *Arch. f. Mikr. Anat. u. Entwick*, 1924, ciii, 437.
‡ Rees, C. W. *Univ. Cal. Pub. in Zool.*, 1922, xx, 333.
§ Davis, H. S. *J. Morphol.*, 1923, xxxvii, 425.
‖ Awerinzew, S. *Arch. f. Protistenk.*, 1909, xiv, 74.
Mavor, J. W. *Proc. Am. Acad. Arts & Sc.*, 1916, li, 551.
¶ Kudo, R. *Trans. Am. Micr. Soc.*, 1921, xl, 161.

ments of spores, therefore, the fresh spores should be measured, or allowance be made for shrinkage.

Microsporidia may be handled by the methods given above for Myxosporidia. For the identification of spores (1) exert pressure on the cover glass over freshly teased out spores to cause the extrusion of the filament. Such extrusion will identify the spores as distinct from bodies of similar size and shape, such as yeast cells. (2) Stain with Ziehl's fuchsin, followed by decolorization with a solution of weak sulfuric acid. Yeast cells are decolorized while spores of Microsporidia remain red.*

Sarcosporidia are found usually as "Miescher's tubes" in the muscles of vertebrate hosts (browsing and grazing animals, mostly). Fix pieces of the tissues and section, or make smears of the tissues. Fix with Bouin's or Flemming's solutions or sublimate-alcohol-acetic. (Saturated solution of sublimate, 75 c.c., absolute alcohol, 25 c.c., and glacial acetic, 15 c.c.), and stain with hematein, hemalum, Mann's methyl blue-eosin, or Heidenhain's hematoxylin followed by eosin and picro-indigo-carmine.†

* Kudo, R. *Biol. Monographs* 9, 1924, Nos. 2 & 3.

† Alexeieff, A. *Arch. de Zool. expér. et gén.*, 1913, li, 521.

CHAPTER IX

FIXATION AND FIXATIVES

C. E. McClung and Ezra Allen

Character of Agent Used 416. Physical agents 416. Chemical agents 416. Differential Effects of Fixatives 426. Selective action 426. Penetration 427. Influence of physical condition 427. Methods of Application 428. By immersion 428. Fixation by injection 429. Relation to After Processes 432. Washing 432. Staining 434.

A. CHARACTER OF AGENT USED

The agents used for the purpose of fixation may be divided generally into physical and chemical, the latter being much the more common.

I. Physical Agents

The only physical agents employed are desiccation and heat.

1. Desiccation. Only infrequently can desiccation be applied, as, for instance, in the smear method recommended by Foot and Strobell (p. 197). Here the fresh tissue is drawn rapidly and steadily across the surface of the glass slip, leaving a thin film behind. Without further treatment the material is then stained and mounted. Not many tissues will return good results with this treatment.

2. Heat. More commonly in smears the protoplasm is coagulated and quite rapidly freed from water by passing it through an alcohol flame. Heat may also be used for large pieces of tissue or even whole animals. In this event the material is placed in water which is rapidly brought near to the boiling point, at which temperature the protoplasm is completely coagulated. Such a method is particularly applicable in the case of insect material. It is only infrequently employed for vertebrate tissues. Except for thin films, the action of a physical agent is less satisfactory than that of a chemical one.

II. Chemical Agents

For the convenience of discussion in part, and also because of distinctive peculiarities, these may be subdivided into purely liquid substances, solutions of solids, combinations of agents, and vapors.

1. Purely Liquid Substances. *a. Examples.* Of the first group may be mentioned:

Acetic Acid. This is one of the most valuable and generally used of reagents, but only infrequently is it employed alone. However, in some instances where it is desired to fix strongly contracting organisms very rapidly, acetic acid is most useful. For best results under these circumstances it may be employed warm. When thus used the action is very

rapid and should be terminated within ten or fifteen minutes. The excess of fixing fluid is washed out with 30 to 50 per cent alcohol and the material preserved in 70 per cent. The general action of acetic acid is to swell the constituents of the cells, and because of this property it is generally combined with some substances which have an antagonistic or shrinking effect. The composition of fixing fluids, in which acetic acid is involved, is therefore a matter of experiment to determine what percentage of the swelling agent should be combined with others which have an opposite action.

For formulas in which acetic acid is combined with other agents, consult the various groups of fixatives.

Aside from the use of absolute acetic acid as indicated, dilutions from 1 to 5 per cent are most useful. It is in proportions like these that acetic acid is combined with other fixing materials. In all dilutions the penetration is good. It is also very valuable because of the strong differentiation which it establishes.

Alcohol. Alcohol, used alone, is employed either as absolute alcohol, or in a strength of approximately 30 per cent. The effective action of absolute alcohol is said to be due to its rapidity of operation, which thereby prevents the distortion of materials caused by rapid combination with their contained water. On the contrary, 30 per cent alcohol is a fairly weak fixative and if applied too long may even act as a dissociating agent for some tissues. For this reason, after fixation of not over twenty-four hours, the tissue should be run up to 70 per cent.

Alcohol is used in combination with other fixing agents especially in the form of absolute alcohol. For these combinations consult the various groups of fixatives.

Chloroform. Chloroform alone is rarely or never employed as a fixative. It finds its chief application in the combinations of Carnoy and of Carnoy-Lebrun (p. 422).

Formic Acid. Formic acid may be used in much the same manner as acetic acid in fixatives but has been found less satisfactory and is not generally employed. An example of the use of formic acid is afforded in the formula by Rabl, which is as follows:

33 per cent chromic acid solution	200 c.c.
Formic acid	5 drops

Fix for twelve to twenty-four hours. Wash in water.

Formol. This substance appears on the market as a 40 per cent aqueous solution of formaldehyde under the trade names of formol, formalin and formolose. It is best to use only the chemically pure solution. The formulas are sometimes ambiguous in not indicating whether it is the strength of the formaldehyde or the percentage of the formol that is meant. For convenience it seems. better to indicate the percentage of the solution rather than of the gas. Solutions decompose after a time, forming paraformalde-

hyde, a white substance. Owing also to the transformation of formaldehyde into formic acid there is often an acid reaction of the solutions. This may or may not be an advantage, but the condition should be recognized. If a neutral solution is required, the addition of an alkali like sodium or magnesium carbonate or even of lithium carbonate, will serve. Formalin may be used alone and gives a fairly good fixation. It is very convenient because no extensive washing is required before sectioning. It has a high degree of penetration and is therefore applicable to large objects. It may be followed by most stains. Formalin is most commonly used in clinical work, when, because of its convenience, rapidity, and general applicability, it lends itself to the conditions of rapid routine work. It may well be questioned, however, whether or not the added precision of the picro-formol-acetic mixtures would not repay the slightly greater time involved in their use.

In using formalin with other substances it should be remembered that formaldehyde is a very powerful reducing agent and therefore rapidly alters the nature of combinations into which chromic acid or other such substances enter. Used alone it is best employed in strengths of 4 to 10 per cent. Fix for several days for best results. Formalin also is a good hardening agent.

Nitric Acid. Nitric acid is not used to any great extent alone as a fixative, but may be employed in a strength of 2 to 5 per cent, and is reported to be valuable because it makes certain tissues brittle.

b. Advantages. These simple fluids have some striking advantages which make them particularly applicable to certain cases. As a rule they penetrate well and rapidly. They leave no deposits in the tissues and they require little or no washing. In cases where rapid results are required these are very obvious advantages. For general routine work various dilutions of formol are much used, as they are also for very resistant objects such as the ova of certain worms and insects.

c. Disadvantages. The very qualities which make these liquids advantageous in some circumstances make them inapplicable in others. Thus, their rapid and vigorous penetration may be injurious to very delicate structures, although under some circumstances they have the opposite effect.

2. Solutions of Solids. These solutions, employed as fixatives, are mostly aqueous in character. Chemically they are either salts or acids. The salts are commonly chlorides of some heavy minerals like gold, platinum and mercury, chromates of the haloid group, and the tetraoxide of osmium. The acids most used are picric and chromic. Solutions of solids alone, without the presence of acetic acid or formol, are rarely employed as fixing agents.

a. Classes. Picric acid is not often used in simple solution, since it shrinks strongly. When combined with other substances it is in strong concnetrations. Dilute solutions of picric acid macerate. Since it forms only a weak

association with the tissues, washing out should be done with alcohol of at least 70 per cent to avoid maceration. When combined with formalin, however, this precaution is not necessary. Since picric acid is also used as a stain it need not always be completely washed out of the tissue if the color is not objectionable. To facilitate the removal of the picric acid, lithium carbonate may be added to the 70 per cent alcohol. Picric acid penetrates well and may be followed by most stains. The simplest method of handling picric acid is to make it up into a saturated solution in distilled water, and it is in this strength that it enters into most combinations.

Chromic Acid (H_2CrO_4), a substance produced when chromic anhydride (CrO_3) unites with water, is rarely used alone as a fixative, but forms a very valuable constituent of numerous mixtures. Combined with acetic acid it is a common fixative of plant tissues, and with the addition of osmic acid appears in Flemming's mixture as one of the most valuable of our cytological fixatives.

The crystals of chromic anhydride are very deliquescent and it is customary to keep the material in aqueous solutions of a strength of 1 or 2 per cent.

Bichloride of mercury is one of the most useful of salts employed in fixing. It has a strong shrinking action and is therefore very rarely used alone, but in combination with acetic acid enters into some of the most commonly used fixatives. The simplest of these is a saturated solution of mercuric chloride in water, to which is added 5 per cent glacial acetic acid. The relative proportions of sublimate and acetic acid should be determined in each case experimentally, adjusting the proportion of acetic acid so as to balance the shrinking action of the sublimate. Sometimes the sublimate is dissolved in alcohol instead of in water and in this way a greater concentration may be obtained. Great care is required in washing out the sublimate from tissues after fixation. If this is not completely accomplished, crystals remain in the tissue which are often of such nature as to be mistaken for normal cell structures. The addition of iodine to the washing fluids, particularly the higher alcohols, facilitates the solution of the bichloride. When the iodine ceases to be decolorized, the sublimate has been completely removed.

b. Advantages. These substances offer a considerable range of effects and, by forming suitable combinations, aid materially in the differentiation of various cellular and tissue structures. In addition they also act as mordants for stains and improve the precision of differential staining.

c. Disadvantages. The disadvantages of these solids in solution are that they may produce deposits, as in the case of mercuric chloride particularly, and they also require a considerable degree of washing in order to remove the uncombined portion.

3. Combinations of Agents in Solution. *General Principles Involved in Choice of Combining Agents.* Such combinations are much more common as fixatives than are single substances. Formulas for these will be given under

the main constituents. It may perhaps be well here, however, to note the general principles which should be followed in forming combinations. It is obviously undesirable to introduce complexity when a simple solution will suffice.

Balancing, Shrinking, and Swelling Effects. In general it may be said that a combination to be effective should balance the swelling action of one reagent by the shrinking effect of another, as when the shrinking action of mercuric chloride or picric acid is counteracted by the swelling action of acetic acid.

Providing a Wide Range of Application by Varying the Proportions of Elements. Because of these antagonistic effects, it is possible, by regulating the proportions of the reagents, to adapt the action of particular combinations to the peculiarities of various tissues. Where three elements are united, the range of application is in some degree further extended. It is only by experimenting with a particular material that an appropriate fixative can be developed. Although we are dependent upon this empirical method, it nevertheless provides a means by which almost any cell element or tissue can be accurately preserved. The range of any one combination is, of course, dependent upon the constituent elements, and some of these are capable of much wider application than others. Of all the combinations so far developed, those containing picric and acetic acids and formol appear to have the greatest flexibility.

Acetic Acid Combinations. Acetic acid is so universal in its use that it enters into nearly all mixtures. Formulas including it will therefore be found under various headings indicating other active constituents with which it is combined.

Bichloride of Mercury Combinations (See also Zenker's Fluid).

(1) *Gilson's fluid*

Nitric acid, 46° strength, about an 80 per cent solution	15 c.c.
Glacial acetic acid	4 c.c.
60 per cent alcohol	100 c.c.
Distilled water	880 c.c.
Mercuric chloride	20 gm.

This is a rather unusual combination but on some materials it produces excellent results. It should be given a trial when difficulties obtain in securing a good fixation with other mixtures. It fixes rapidly but does not commonly overfix, and in the case of amphibian eggs, if allowed to act for several days, will dissolve off the albumen.

(2) *Worcester's fluid*

10 per cent formalin saturated with mercuric chloride	9 parts
Glacial acetic acid	1 part

This is an excellent fixative which deserves wider use.

(3) *Heidenhain's trichloracetic with sublimate**

Saturated aqueous solution mercuric chloride	100 parts
Trichloracetic acid	2 parts
Glacial acetic acid	1 part

(4) *Formol-sublimate*

10 per cent formol saturated with mercuric chloride

(5) *Sublimate acetic*

Saturated aqueous solution of mercuric chloride	95 parts
Glacial acetic acid	5 parts

Sublimate fixatives are rapid and vigorous in action and are best used on small objects for short periods of time. With most stains they give sharp and brilliant pictures. They require careful washing, which may be facilitated by the presence of iodine in the higher grades of alcohol.

Bichromate Mixtures. The original of these combinations is Mueller's fluid. This is prepared as follows:

(1) *Mueller's Fluid.*

Potassium bichromate	25 gm.
Sodium sulphate	10 gm.
Water	1000 c.c.

This is now rarely used for fixing, but is an excellent hardening agent. There seems to be no reason for the addition of sulphate of soda and it might just as well be omitted.

(2) *Zenker's Fluid.* This is a modification of Mueller's fluid, produced by adding to it 5 per cent of mercuric chloride and 5 per cent of glacial acetic acid. Fix ten to twelve hours; wash with water. The sublimate may finally be removed by adding iodine to the washing fluid, preferably to the alcohol in which the sections are dehydrated. Too long a fixation produces deposits of crystals which are difficult to remove.

(3) *Helly's Fluid; or Zenker-formol.* This is a modification of Zenker in which the acetic acid is replaced by 5 per cent formalin. This fixative gives very beautiful results but in some materials extensive distortions are produced when paraffin sections are made. The same material cut in collodion gives very beautiful and precise figures. It is particularly recommended for blood work, both on tissues and smears.

(4) *Tellyesniczky's Fluid.*†

Bichromate of potash	3 gm.
Water	100 c.c.
Glacial acetic acid	5 c.c.

Fix twenty-four to forty-eight hours. Wash in water.

* Heidenhain, M. *Ztschr. f. wiss. Mikroscop.*, 1909, xxv, 405.

† Tellyesniczky. *Arch. mikr. Anat.*, 1889, xxxiv, 52.

Alcohol Mixtures.

(1) *Carnoy's fluid*

1. Glacial acetic acid ..... 1 part
   Absolute alcohol ..... 3 parts

2. Glacial acetic acid ..... 1 part
   Absolute alcohol ..... 6 parts
   Chloroform ..... 3 parts

(2) *Carnoy-Lebrun*

Absolute alcohol ..... 1 vol.
Glacial acetic acid ..... 1 vol.
Chloroform ..... 1 vol.
Saturate with mercuric chloride

This group of fixing fluids is an extremely valuable one because of the very good penetrating power possessed. Such difficult objects as eggs of Ascaris megalocephala and of insects are best fixed with these reagents. For cytological studies of nuclear structures, with the picro-formol-acetic mixtures and chrom-osmium-acetic combinations, they constitute a resource of almost universal application. Fixation is rapid. Wash in 95 per cent alcohol.

Chromic Acid Mixtures. The chromo-acetic combination given by Flemming consists of 25 per cent of chromic acid and 1 per cent of acetic acid in water. A stronger solution consists of

1 per cent aqueous solution of chromic acid ..... 95 parts
Glacial acetic acid ..... 5 parts

(See osmic acid mixtures for formulas in which chromic acid appears, p. 423.)

(1) *Destin's fluid*

1 per cent aqueous solution of chromic acid ..... 99 c.c.
Formol ..... 6 c.c.
Glacial acetic acid ..... 2 c.c.

Allow the brown fluid to stand a few days until it becomes green before use.

Nitric Acid Mixtures. The most frequently used combination of nitric acid is that of Perenyi which consists of nitric acid in 10 per cent solution, 4 parts; alcohol 95 per cent, 3 parts; chromic acid, 1 to 2 per cent, 3 parts. This is in some cases a very valuable fixative but seems rather uncertain in its reaction, possibly because of chemical changes which it undergoes on standing.

(1) *Formol-nitric.* A combination which has proved very valuable, especially in the fixation of chick embryos, is the following:

Formol, 10 per cent ..... 3 parts
Nitric acid, 10 per cent ..... 1 part

This is best applied by opening the shell of the egg, removing the superficial albumen, and then adding the fixative directly to the embryo. After ten minutes the blastodisc may be cut out with scissors and removed to a watch glass where it is hardened with formol sublimate.

Osmic Acid Mixtures.

(1) *Flemming's fluid.* Strong formula

(a) Chromic acid, 1 per cent aqueous solution	11 parts
Glacial acetic acid	1 part
Distilled water	4 parts

(b) Osmic acid, 2 per cent in 1 per cent chromic acid solution.

Just before using mix 4 parts of (a) with one part of (b). Use 10 times the volume of the object. Fix two to seventy-two hours. Wash in water twenty-four hours.

A precise and delicate fixative for cytological details and adapted to many kinds of protoplasm. It may in some cases be used to advantage at low temperatures—0°C. for instance. Does not penetrate deeply and sometimes overfixes on the periphery of the mass and underfixes in the center. May be used advantageously when staining in iron hematoxylin or with aniline dyes (see also p. 461).

(2) *Lillie's chrom-osmic-acetic mixture*

Chromic acid, ½ per cent solution	15 c.c.
Osmic acid, 2 per cent aqueous solution	3½ c.c.
Glacial acetic acid	3 drops

This is recommended by Lillie for fixing echinoderm eggs in order to follow the exact changes during fertilization.

(3) *Bichromate-chromic-osmic acid mixture of Champy:**

3 per cent aqueous solution of bichromate of potash	7 parts
1 per cent chromic acid aqueous solution	7 parts
Osmic acid, 2 per cent aqueous solution	4 parts

Fix six to twenty-four hours. Wash in water for same period of time. Stain with iron hematoxylin.

(4) *Platino-aceto-osmic acid mixture of Hermann.*†

Platinic chloride, 1 per cent aqueous solution	15 parts
Glacial acetic acid	1 part
Osmic acid, 2 per cent aqueous solution	2 parts

Fix twelve to twenty-four hours. Wash for similar period of time in water. This in effect is very similar to Flemming's fluid. For a nuclear stain follow with iron hematoxylin. Chromosomes appear larger upon fixation in Hermann's fluid than in Flemming's. It is more difficult to secure a good cytoplasmic stain than after Flemming.

* Champy, C. *Arch. de zool. expér. et gén.*, 1913, lii, 13.

† Hermann, F. *Arch. f. mikr. Anat.*, 1889, xxxiv, 81.

Platinic Chloride Mixtures.

(1) *Juel's fluid*

Chromic acid, 2 per cent aqueous	25 c.c.
Platinic chloride, 10 per cent	2.5 gm.
Glacial acetic acid	1 c.c.
Water	75 c.c.

(2) *Merkel's fluid* (F. E. V. Smith's modification)

Acetic acid, 5 per cent	100 c.c.
Platinic chloride 1 per cent	5 c.c.
Chromic acid, 1 per cent	10 c.c.

Picro-formol-acetic Mixtures Used at the University of Pennsylvania.

(1) *Bouin's fluid*

The original picro-formol-acetic combination is that of Bouin. It is prepared as follows:

Saturated aqueous solution of picric acid	75 parts
Formol, C.P.	25 parts
Acetic acid, glacial	5 parts

(2) *Modifications of Bouin.* A series of experiments was carried out at the University of Pennsylvania, using a large number of combinations of the three elements contained in Bouin's fluid, to which other substances were added. These were employed particularly for the finest cytological details and it was found that some improvements could be effected by modifications of the original formula. Those of most value have been described in the literature as P.F.A.$_3$ and P.F.A.$_{15}$ and, when combined with chromic acid, are marked with the designation B-3 and B-15. Preparation is as follows:

(a) *Allen's fluid* P.F.A.$_3$.

Picric acid, saturated aqueous solution	75 parts
Formalin, C.P.	15 parts
Glacial acetic acid	10 parts
Urea	1 part

(b) B$_3$ consists of this fluid to which is added chromic acid, 1 part.

(c) P.F.A.$_{15}$ (B-15) is the original Bouin's fluid to which are added two parts of urea and 1.5 parts of chromic acid.

The fluids with chromic acid turn green in perhaps half an hour, and after this change takes place they do not seem so efficient. The purity of the chemicals is extremely important. If a precipitate forms upon the addition of the urea, either it has not been stirred in carefully, or the formol is impure. If the color is blackish instead of a deep reddish brown the trouble is probably with either the formol or the chromic acid.

The addition of chromic acid to the mixture requires that it be used immediately, since rapid reduction of the chromic acid is effected by the formol. It is best, therefore, to add the chromic acid only at the time that

fixation takes place. The P.F.A. mixtures without chromic acid are very valuable since material may be left in them for long periods of time without danger of overfixation. This is particularly true of the combination P.F.A.$_3$ and it has been found in the case of Orthopteran material that whole animals, which have been opened sufficiently for it to enter readily, may be so treated that the intimate cytological details of the cells, the germ cells particularly, are well preserved. This is a very great convenience when rapid preparation in the field is necessary. (For further discussion of modified P.F.A. mixtures see p. 184.)

	Bouin	B-15	3	B-3	2	8	16
Picric acid sat. aq. sol.	75	75	75	75	75	90	50
Formol	25	25	15	15	10	5	20
Acetic acid, glacial	5	5	10	10	10	5	5
Urea	..	2	1	1	..	..	1
Chromic acid	..	1.5	..	1	..	..	1
Water	..	..	..	..	..	..	25

(3) *Picro-sulphuric acid**

Distilled water ..... 100 vol.
Sulphuric acid ..... 2 vol.
Saturate this mixture with picric acid.

This was formerly much used in embryological work, but it has been found less desirable than the picro-formol-acetic mixtures.

(4) *Picro-acetic*†

Saturated aqueous solution of picric acid ..... 100 parts
Water ..... 200 parts
Glacial acetic acid ..... 3 parts

4. Vapors. For very delicate objects, present in a small quantity of water or disposed as a thin film, fixation may be secured by exposure to the vapors of certain substances. Principally available here are formaldehyde and osmium tetraoxide. To secure action in this manner it is sufficient to bring the object into a small chamber containing the gaseous reagent. Films on cover glasses are conveniently inverted over a vessel containing a solution from which the gas arises. In the case of osmium tetraoxide a few drops of Flemming's fluid in a small Stender dish will provide a ready means of fixing smears on covers. When it is desired to fix objects under observation this may be done by observing them on an inverted cover over a moist chamber into which the vapor may be drawn when desired.

* Kleinenberg. *J. Mikr. Sci.*, 1879.

† Boveri. *Sitzungsb. d. Gesellsch. f. Morphol. u. Physiol.* 1886, ii, 101.

Fixation by vapors is a precise, delicate and ready method when it is applicable.

B. DIFFERENTIAL EFFECTS OF FIXATIVES

These may be considered under several headings:

I. Selective Action

There is the question of their selective action. There seems to be no good reason for classifying fixatives as cytological, histological, or embryological in application because, with good powers of penetration, well-balanced combinations such as the picro-formol-acetic mixtures, will preserve cells, tissues, or organs almost equally well. However, there do exist certain selective actions between parts of cells and particular reagents, so that for certain cytoplasmic structures ordinary nuclear fixatives are regarded as unsatisfactory. Such differences nevertheless may, in part at least, be the result of method of after-treatment. It is commonly stated that the presence of acetic acid in any combination is inimical to the preservation of mitochondrial structures. It has been found by experiments on Orthopteran spermatocytes that Flemming's fluid, if only lightly washed out, is a fixative which, followed by hematoxylin, presents an almost perfect mitochondrial stain. The elements have not been removed by the acetic acid, as supposed, but commonly after long-continued washing when a good nuclear stain results, the mordanting action of the fixative has been suppressed and the mitochondria do not retain the hematoxylin. The experiment by which this demonstration was carried out is as follows:

A Flemming-fixed preparation of grasshopper testis, after the sections were spread and dried, had the paraffin removed from one end of the slide by xylol. The whole preparation was then placed in 70 per cent alcohol for several days, after which the paraffin was removed from the remaining sections and the whole preparation stained with iron hematoxylin. On examination, the washed-out sections were found to have clear, stained nuclei with no mitochondria showing, while those which had been protected by the paraffin and were only slightly washed showed a reverse staining reaction with the mitochondria dark and the chromatin unstained. It is thus apparent that the selective action of any fixative is in a measure dependent upon the after-treatment of the material.

Taking all of these facts into consideration, therefore, as a practical matter, the differentiation of fixatives into nuclear and cytosomic is justified. We may well question, however, whether the effects produced upon the cytoplasmic elements are due to the absence of acetic acid or to the mordanting action of other substances in the mixture. Most of the methods adapted to mitochondrial or Golgi apparatus constituents require extended action of the fixatives, even at high temperatures, both

of which conditions facilitate mordanting action. In general, unless stated otherwise, the fixatives given are adapted to observation of nuclear structures, and those employed for cytoplasmic constituents are separately considered (see also Cytological Methods, p. 198).

II. Penetration

Further differential effects may be due to the rate and degree of penetration of the agents in the fixative.

1, 2. **Rate and Degree.** These are particularly marked in the case of Flemming's fluid. The acetic acid enters rapidly and well, while the osmic acid is restricted in its action to the superficial layers of cells. Because of this difference in character of the penetration, a piece of tissue presents very often a peripheral zone in which the protoplasm is glassy and homogeneous while the central portion stains with characteristic differentiation between the cell elements. In any combination, therefore, it is necessary to know the physical characteristics of the elements which enter into it if the best results are to be obtained from its use.

III. Influence of Physical Condition

The differential effects are also due to the physical condition of both the specimen and the medium.

1. **In Specimens.** *Size and Density.* Conditions of the specimen which influence action of the fixative are size and density. In choosing a fixative, therefore, the nature of the tissue must be carefully studied. Very dense or resistant substances require reagents of vigorous action and rapid penetration, whereas loose tissues may be fixed with comparatively mild ones.

2. **In Medium.** The physical state of the medium in regard to temperature concentration and the presence of adjuvants, has much to do with the differential effect of the fixative. It has long been recognized that elevation of temperature facilitates rapid penetration, but it has not been so well recognized that this produces a difference in its action on various cell elements. In a series of experiments conducted at the University of Pennsylvania it was discovered that the relative density of nucleus and cytoplasm may be varied by the temperature of the fixative.

a. Temperature. In extreme cases the nucleus appears practically empty, because of the solvent action of the fixative, but by the application of Flemming's fluid at very low temperatures, in the presence of urea, the nucleus of grasshopper spermatocytes is considerably denser than the cytoplasm. Apparently a better cytological fixation is secured by the use of Flemming's fluid at 0°C., while picro-formol-acetic combinations do better at a temperature of 35 to 40°C.

b. Concentration. The concentration of the fixative, as a whole, as well as the proportion of its parts, also influences its selective action. Only very recently, however, has it been realized that the presence of relatively inert

substances, such as urea, various sugars, etc., has a pronounced influence upon the operation of the fixative. The action of urea has already been referred to, it being the first of such substances employed. Some experiments upon plant cells with different sugars indicate that they have specific effects. For instance, glucose added to Flemming's fluid produces in each cell of the Podophyllum root tip a large clear vacuole, whereas if levulose is substituted for the glucose no such vacuole is seen.

It thus appeared that these sugars, having such specific actions, might be advantageously employed in the fixing of plant tissues. An extension of these experiments was therefore carried out in the Botanical Laboratory, University of Pennsylvania, and will be found discussed more fully in the chapter on Botanical Methods (p. 148).

c. Adjuvants. So far, the studies upon differential action of adjuvants in fixatives have been of a preliminary sort, but they indicate strongly the value of such substances and the need for a more complete investigation of their action. Some early studies by McClendon* showed that the swelling and cytolysis of cells in the convoluted tubules of the kidney could be prevented by adding 10 to 40 per cent of sugar to the fixative. The small percentage of urea and sugar employed in our experiments would not seem to operate by affecting the density of the reagent, as in McClendon's experiments, and we can only suppose that some more exact adaptation is involved in their specific effects. Since these are substances which penetrate protoplasm with unusual rapidity it may be surmised that their action is, in part at least, due to this property.

C. METHODS OF APPLICATION

The methods of application of fixatives may be considered in relation to the kind of material. Small pieces of tissue may be immersed directly in the fixing fluid, whereas if large organs or whole animals are to be fixed, the reagent should be injected through the circulatory system.

I. By Immersion

If fixation is by immersion, regard must be had for the following circumstances:

1. Proportionate Volumes. The volume of the fixing fluid in relation to that of the tissue must be such that the water abstracted from the tissues will not so dilute the fixative as injuriously to affect its action. This proportion varies with the particular fixative employed. In general there should be a volume of the fixing fluid 25 to 50 times that of the object. It is also desirable sometimes to substitute fresh fixative, especially if it is applied for considerable periods of time.

2. Position of Specimen in Fluid. The position of the specimen in the fluid is not unimportant. If it is possible to suspend the object near the

* McClendon, J. F. *Anat. Record*, 1913, vii, 51.

surface of the medium it is advantageous, because in this position it is always in a reagent of high concentration. If the specimen has been attached to a small piece of paper it will float on the surface unaided. Larger objects may be suspended by a thread in a cylindrical vessel.

3. Duration of Action. The duration of action required varies with the material and the particular fixative. There are some reagents which produce injurious effects by overfixation, whereas others may be allowed to act indefinitely. The minimum time for effective results can be determined only by experiment, but as a rough guide it may be said that a piece of tissue not over 5 mm. in diameter in chromic acid combinations will be fixed within a period of two to twelve hours. The picro-formol-acetic combinations (without chromic acid) may be allowed to act for almost any length of time without injury to the specimen. Rapid and vigorous fixatives, such as those of Carnoy, fix within an hour in some cases.

II. Fixation by Injection

If the presence of blood is not essential, a better picture of the relationships of all other tissues to each other, as well as of chromosomes, may be obtained in complex organs, as brain, testis, etc., by injecting the fixing fluid rather than by cutting the organ into small pieces. In the case of testes which have a small quantity of interstitial tissue, as the rat's, this tissue is not torn.* Any fixative may be injected.

The blood must first be removed by washing out the vessels with Locke's or normal salt solution, which for mammals is 0.9 per cent. The washing is begun while the animal's heart is still beating, if possible; consequently light etherization is essential. As soon as the blood is out of the veins the fixing fluid is injected. Each fluid may be forced in by gravity or by air pressure. Too great pressure is to be avoided throughout the process, as extravasation is easily produced. A pressure equal to about 10 mm. of mercury is sufficient for a white rat. It may be obtained through gravity by elevating the container of the fluids to a height of about 25 to 30 inches. A funnel, supported on a retort stand, answers the purpose fairly well. This is connected to the cannula by rubber tubing. A screw clamp will control the rate of flow. Some workers advocate a pulsating flow, imitating the heart beat, but the writer has found a steady flow perfectly satisfactory.

A more elaborate apparatus, by which the pressure may be varied, is shown in Figure 1 (p. 189). This consists of a pressure bottle A, and an aspirator bottle B for holding the fluids. The delivery tube D.T. from B carries the fluid through the warm water in C to the cannula. The flow is controlled by the screw clamp *d*. In order to free the fluid from any air bubbles which might stop the flow of the fluid in the blood vessels, a vent is introduced by the T-tube, controlled by clamp *b*. The atomizer bulb is used to force air into A. The resulting pressure is communicated to the

* Allen, E. *Anat. Record*, 1919, xvi, 25.

fluid in B through the connecting tube *c.t.*, and controlled by the screw clamp *a*. The mercury manometer registers the pressure.

Instead of the atomizer bulb, one may use a bottle of water elevated about 2 feet and connected by tubing to A, as shown in Figure 2 (p. 189). The Woulff bottle may be replaced by a salt mouth bottle fitted with a three-hole rubber stopper. Or, if air pressure is supplied in the laboratory, it may be used under proper control. In this case, a manometer is absolutely essential. If desired, 2 B bottles may be used. A Y-tube will connect both to the delivery tube; flow from each is controlled by a screw clamp. For use, disconnect the tube *c.t.* from the bottle containing the salt solution and connect with the other. Use rubber stoppers and heavy walled tubing throughout except for the short cannula tube, where flexibility is desirable.

1. **Steps in Injecting.** *a. Preparation of Apparatus.* Whatever form is decided upon, set it up fully and test it thoroughly before preparing the animal. Have several cannulas varying somewhat in size. If possible, try the cannulas on an experimental animal to be sure the right size is at hand. Have ready means of keeping the animal at body temperature. For this purpose use a vessel of warm water under a dissecting pan, or a waterproof electrically heated pad or table. Have salt or Locke's solution and the fixing fluid at the right temperature, and be sure they can be held at that temperature. As there always is some loss of heat in the tubing, 40° to 45°C. is about right. When entering a mammal each fluid should be at about 37° or 38°C. As a source of heat a paraffin oven or a water bath is much better than a gas burner, as the latter must be watched lest it overheat the fluid. The fixing solution should be well stoppered while being heated and while in use. Have something to place under the animal's shoulders, as working in the chest cavity is much easier if it is elevated. The head may droop. Have dissecting tools ready and in place.

b. Washing. Having everything ready and the fluids at the proper temperature, let the salt solution flow down into the cannula while the animal is being etherized. Get rid of all air bubbles by letting them escape through the T-tube. Let a very slight flow continue, enough so that the cannula will be full when held in any position. This flow will not interfere with its insertion into the blood vessel, and guards against clotting.

Lay the animal on its back, the heart still beating, and make a longitudinal midventral incision through the skin from the genital outlet to the neck. The hind legs may be tied fast and the front legs tied behind the back. Slit the abdominal muscles to the breastbone, and cut the ribs a little to the left of the breastbone with care not to cut the mammary artery. Bend the ribs back, breaking them if necessary, and even removing the ventral ends, to have a clear cavity in which to work. The slight loss of blood is of no consequence. Cut the left side of the diaphragm from the ribs. The heart should still be beating. If the whole animal or only the

anterior end is to be used, inject through the heart. If only the hinder part is wanted insert the cannula in the thoracic aorta.

Suppose the testes of a rat are to be fixed with warm B-15. After securing the cannula firmly in the thoracic aorta, start the flow of salt solution with a very gentle pressure, merely sufficient to distend the artery posterior to the cannula. Stop the pressure by closing the valve *b*, and open the posterior vena cava to let the blood escape. It may be absorbed in the cavity by absorbent cotton, but keep it loose to avoid back pressure. Now turn on the pressure gradually but quickly to 20 or 25 mm. mercury counting the movement in both arms of the manometer. The testes should be in the scrotum. If retracted, push them back. They do not seem to inject well if in the body cavity. Slit open the scrotum on one side to observe the loss of blood in the prominent testicular vessel. Watch the intestines and the liver. By the time this last-named organ is pale the blood is about washed out. If these organs do not pale, the flow is interrupted. If one testis is pale, the other is almost certain to be washing satisfactorily if it is in its natural position in the scrotum.

c. Injecting the Fixative. To change to the fixing fluid, close the clamps *d* and *a*. If two aspirator bottles have been provided, close the clamp for the salt solution, change the connecting tube to the other bottle, open its discharge clamp and clamps *a* and *b*. Start the pressure gently, and continue until the salt solution in the delivery tube has all been replaced by the fixing solution, and all air bubbles have escaped; then close clamp *b* and open *d*. Increase the pressure as before. In this case the fixative is colored, and the exposed testis, the intestines and the liver should quickly appear yellow, and the fixative should escape from the vena cava. Continue the flow until about 100 c.c. have passed through. Let more through if the flow still continues. Toward the latter part of the process, the pressure may be increased slightly without injury to the tissues. The hinder parts of the animal will be stiffened by the formol. To insure preliminary fixation in a rat 100 c.c. is sufficient.

d. After-treatment before Dehydration. Remove the testes, still covered with the tunica albuginea, and also the epididymis if desired, and place in fresh fixative at 38°C. After about thirty minutes slice the testes with a thin safety razor blade into 3 or more pieces, preferably longitudinally, and return the tissue to the fixative for another half hour. Longer fixation in this fluid is undesirable for these organs. They may be placed in Bouin if for any reason the dehydration process is to be delayed.

e. Injecting through the Heart. In this case a larger cannula is used. Inject through the left ventricle for either the anterior end or the whole animal. If the lungs are not desired, let the blood escape from the right auricle; if they also are to be injected, clamp the opening in the right auricle, removing the cannula to the right auricle. Two animals may be needed for both purposes if they are small.

If a colorless fixing fluid is to be used, a little eosin will serve to mark its progress, unless the fixative decolorizes the eosin rapidly.

D. RELATION TO AFTER PROCESSES

I. Washing

1. Medium. The selection of the medium for washing is determined by the nature of the fixative employed. It is always emphasized that after picric acid combinations the tissue should not be allowed to remain long in water because of the macerating effect which it exercises, whereas the chromic acid combinations are so vigorous in action that water will not injure the specimens during a period of even twenty-four hours. As a general rule it may be noted that the sooner the material is brought into 70 per cent alcohol the better. Most of the reagents are soluble in alcohol, so that after a preliminary washing the specimen can be run up into that grade and by changing this repeatedly the excess fixative may be removed. Certain combinations not aqueous in their nature, such as Carnoy's mixtures, should be run into an appropriate grade of alcohol at once without passing through water. The rule is to proceed from the fixative into a concentration of alcohol somewhat similar to that of the fixative.

2. Method of Application. It is sometimes sufficient to place the fixed material directly in the washing medium in any convenient vessel, but where small objects are handled, especially in large numbers, it is often helpful to utilize special pieces of apparatus. A number of these have been devised and consist either of small vessels having perforated bottoms or cylinders capped with porous fabric. By causing a current of water to flow through, the specimens are washed rapidly without danger of loss. The following is a description of such an arrangement by Allen:*

Automatic Device for Changing Fluids on Many Small Objects

The apparatus (Fig. 1) is essentially a glass tube about 300 mm. long by 16 mm. inside diameter, supported at an angle of about 30°, fitted with a glass stop-cock at the lower end and with a 2 hole rubber stopper at the upper, through which fluids are conducted from the supply bottle. The overflow control tube fitted into the upper stopper serves two purposes: when the large glass tube is being filled from the supply bottle, it allows air to escape; it prevents overflow when the tube is full and the stop-cock is closed. A retort stand fitted with rings and clamps is adequate support. The supply bottle is of the aspirator type. The wads of cotton (cotton and c) insure the thorough mixing of the new fluid with that in the tube. The upper wad (c) should be on top of the tissue; the space between it and the intake gives opportunity for mixing before the new fluid reaches the tissue. If tissues are inclosed in small glass tubes, each with its number, and covered with wide-meshed cloth, it is well to place the tubes in lengthwise, and not to allow them to be tightly packed.

*Allen, E. *Science*, 1927, lxvi, 427.

Tissues may be fixed, stained, washed, dehydrated, and cleared without removing them from the tube. The process may be interrupted at any step, and the tissues left in the appropriate fluid overnight or longer without

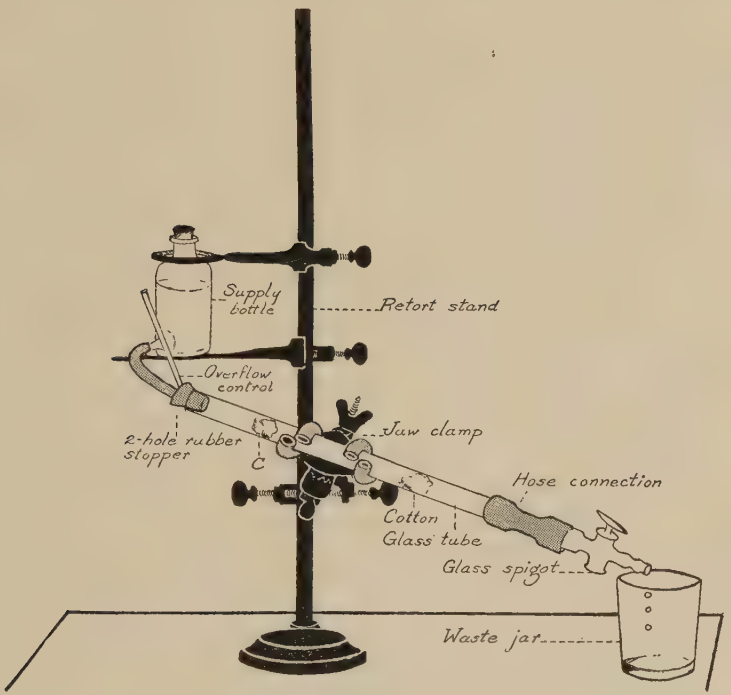

FIG. 1.

loss by evaporation. Simply close the spigot. The rate of change is controlled by the rate of dropping. Used fluids may be preserved, account being kept on the labels of the number of times used. One may introduce 50 per cent alcohol into water; 95 per cent into 50 per cent; but it is better to use intermediate mixtures if cytological details are especially desired.

3. Duration and Extent of Action. The washing-out processes must be determined by the effect desired. Commonly it is best to remove all of the uncombined material, but sometimes for mordanting purposes, as indicated in the discussion of mitochondrial stains, the washing should not be complete. In general, if complete extraction of the uncombined fixative is desired, the length of the period should approximate that of the action of the fixative. If the medium employed is water, however, this should not extend beyond twenty-four hours as a rule.

II. Staining

1. Mordant Action. As has been indicated in the discussion of the process of washing, fixation bears a definite relation to the after-process of staining. After most fixatives almost any nuclear stain may be applied, although there are differences in the degree of precision of the stain according to the nature of the fixative. The mercuric bichloride combinations show a very brilliant reaction with hematoxylin stains. Certain of the aniline dyes do not operate well unless osmic or chromic acid has been present in the fixative. A further discussion of this subject will be found under the heading "Stains" (p. 461).

2. Revival of Staining Capacity. The condition in which cells are left by the fixing fluid is not stable. From the point of view of staining possibilities a slow deterioration sets in immediately, and the thinner the tissue the sooner a loss of staining power is noticeable. Thus, a block of tissue may remain in good condition for years, while a smear or connective tissue spread is less satisfactory in a few days. If the fixation was good in the first place and the deterioration is not too pronounced, staining capacity can be revived by the use of peroxides. Smears, membranes, or sections not more than 10μ in thickness should be placed in commercial hydrogen peroxide for thirty minutes or more and then washed in water before being stained. A 10 per cent solution of benzoyl peroxide* (St. Szecsi, p. 247) in acetone is even more satisfactory and requires only fifteen minutes. It should be washed out in xylene-acetone (2 to 3), followed by alcohol. In the case of a faded slide which is worth restaining, it is advisable to soak off the coverglass and treat the preparation with benzoyl peroxide solution before putting it into the stain (Slider).

* Benzoyl peroxide is unstable if heated.

CHAPTER X

STAINS AND STAINING

C. E. McClung and H. J. Conn

Nature of Staining Agent 435. Origin or source 435. Aniline dyes 437. Composition of staining agents 461. Nature of Staining Combination 462. Physical nature 462. Chemical nature 463. Application of Stains 463. Intra vitam 463. Post mortem 463. Formulas 466. Aniline dyes 466. Carmine and Cochineal 467. Hematoxylin and hematein 468.

A. NATURE OF STAINING AGENT

I. Origin or Source

1. Organic. *a. Natural.* Natural organic dyes are (1) from an animal source (carmine) and (2) from plants (hematoxylin and brazilin).

Carmine and Cochineal. Carmine is the only animal product used in staining. Certain scale insects, particularly coccus cacti coccinillifera, secrete a colored substance, cochineal, which is used commercially as well as scientifically. The dried bodies of these insects are ground up into a powder and in this condition may be dissolved in water to which is added either an acid or an alkali, thus forming directly a staining solution. Usually a plain aqueous solution is not employed, but has added to it certain adjuvants as in the case of hematoxylin.

While stains may thus be made directly from the crude material, in general the derived substance, carmine, is the basis of the stain. Carmine, however, is not a simple chemical compound but a complicated mixture of which the essential coloring agent is carminic acid. Therefore instead of using either the crude cochineal or the product obtained from it, carmine, the active staining principle, carminic acid, may form the base of the stain.

Carmine is a bright red substance. Cochineal inclines more to an orange red. Of all stains, these are most applicable for staining in toto because they do not overstain. Particularly in embryological work, the cochineal stains, applied before sectioning, are of very great value. Carmine stains in general are used progressively. As solvent media both water and alcohol are employed. Also they may be either acid or alkaline in reaction. Carmine is primarily a nuclear stain, but properly applied may be made to color selectively various parts of the cell.

Brazilin ($C_{16}H_{14}O_5 + 1\frac{1}{2}H_2O$) is a natural dye obtained from Brazil wood, Cesalpina crista. It is similar in general character to hematoxylin, but has a red color. It is not so active and strong a dye as hematoxylin and has had only a limited use. It is possible that more careful investigation of this dye might disclose more specific application than it has received. It may be handled in the same manner as hematoxylin, as either a progressive

or a regressive stain. Like hematoxylin, brazilin acts in staining through the presence of an oxidized form—brasilein, $C_6H_{12}O_5$.

Hematoxylin ($C_{16}H_{14}O_6$), is derived from logwood, Campechianum. It is best obtained by extracting the wood with water in the presence of ether. Usually, however, it is prepared by taking the logwood extract which is found in commerce and treating it with ether. The process is a rather long and difficult one and for some reason the products of successive operations differ in their physical characteristics. Whether there is any chemical variation is not known. As found on the market hematoxylin is in the form of small crystals, sometimes acicular. Some samples on the market are very white, a result produced by using sulphur dioxide in the process of manufacture. This practice is undesirable, since it injuriously affects the keeping qualities of solutions, and it is being abandoned. Commonly crystals range in color from light yellow to a rusty purple. In the dry condition the material keeps indefinitely. If combined with one molecule of water the crystal form is rhombic, with three molecules it is tetragonal.

Hematoxylin is soluble in water, glycerin and alcohol. It is commonly used in aqueous solutions either plain or combined with a mordant. Aqueous solutions are most readily prepared by first dissolving the hematoxylin crystals in a small quantity of 95 per cent alcohol and then adding water in sufficient quantity to make the solution of the right concentration, usually ½ per cent. Such solutions do not keep indefinitely and during the development of American hematoxylin much difficulty has been encountered in preparing it so that it would remain usable for any length of time. It has been found that the addition of a very small quantity of a sulphite, such as sodium bisulphite, will preserve the solution in good condition very much longer. This prevents oxidation, of course, which is not a drawback in Heidenhain's technique. In case such a solution is employed in a progressive stain the preservative action of the sulphite must be overcome by some oxidizing substance.

Hematoxylin does not stain directly, but only after it has been oxidized to hematein, and in stains operating progressively this process must occur before use. In most cases this is accomplished by allowing it to "ripen," which is a process of slow oxidation. It may be more readily and accurately accomplished by the addition of a small quantity of hydrogen peroxide to the solution. The color depends upon the degree of oxidation, hematein, $C_{16}H_{12}O_6H_2O$, producing a blue color while dioxyhematein, trioxyhematein and tetraoxyhematein are progressively browner. Apparently the best form for use is the trioxyhematein. The higher compounds are less precise in action and less agreeable in color.

In regressive stains, such as Heidenhain's iron-hematoxylin, the tissue is first mordanted. Subsequently, when immersing it in a hematoxylin solution, a lake is formed between the mordant and the staining agent and this becomes fixed in the elements of the tissue.

Hematoxylin is not a good agent for in toto staining, but it is probably the very best of our dyes for sections. It may be applied either progressively as in the case of Delafield's hematoxylin, or regressively in the classical iron alum-hematoxylin method of Heidenhain.

The color effects in tissues produced by the application of hematoxylin vary with the character of the medium in which it is dissolved. In the presence of acids the color is red. In the presence of alkalies it is blue. Commonly, because tap water in which sections are washed is slightly alkaline, the final result is blue. Fresh solutions, with their lower oxidation products of hematein, give bluer effects than the same ones after use. Finally, in old baths, only a rusty, greenish black color results. In neutral balsam, unchanged by age, the color of hematoxylin in sections is permanent. Of all the stains available to the microscopist, hematoxylin is the most generally useful because of its power, selectivity, precision and permanence, in addition to its pleasing color. Because of its particular affinity for chromatin with ordinary fixations and for cytoplasmic constituents under modified techniques, it is invaluable in cytological studies. It is also very adaptable to different mordants and may be combined with various compounds containing aluminum, iron, chromium, molybdenum, etc., to form stains for specific purposes.

b. Synthetic Dyes. Aniline dyes. These are classified, according to their action on tissues, as basic and acid. Basic anilines are the ones having the staining radical in the position of the base in a salt and they therefore operate upon the acid parts of the cell like the chromatin and nucleus. Acid dyes, on the contrary, are those in which the radical is in the position of the acid in the salt. There are a great many aniline dyes, but for most purposes a limited number are entirely satisfactory. The basic aniline colors most in use are Bismarck brown, cresyl blue, fuchsin (basic), gentian violet or crystal violet, Janus green B, methyl green, methylene blue, neutral red, safranin, thionin, toluidine blue. The acid dyes most in use are Bordeaux red, Congo red, eosin, fuchsin (acid), orange G, Sudan III, trypan blue. A full discussion of the characteristics of aniline dyes by Conn follows.

Aniline Dyes*

Outstanding information concerning the nature and uses of biological stains has been gathered together here. Very little information is given concerning the chemistry of the dyes, and nothing concerning the technique by which they are employed. For the former information one should consult other authorities; for the latter, other sections of this book.

The dyes are listed below in alphabetical order, as this seems the arrangement by means of which the biologist can most readily find any dye he may desire. The confusing synonomy of dyes often has made it necessary to list the same dye in several places; but the description of the

* H. J. Conn.

dye is given only under the one name which has been adopted by the Commission on Standardization of Stains as the preferred designation.

For further detail along the same line as given below the worker is referred to the book "Biological Stains."* For technique the other sections of this book may be consulted, and also such standard texts as "Krause's Enzyklopädie,"† Lee's "Microcomist's Vade-Mecum,"‡ and Mallory and Wright's "Pathological Technic."§

Acid Bordeaux. See Bordeaux red.

Acid Congo R. See vital red.

Acid Fuchsin. See fuchsin, acid.

Acid Green. See light green SF, yellowish.

Acid Magenta. See fuchsin, acid.

Acid Orange. See orange II.

Acid Rubin. See fuchsin, acid.

Alcohol Soluble Eosin. See ethyl eosin.

Alizarin. An acid dye of the oxyquinone group, formerly extracted from madder root, but now prepared synthetically. It stains tissues a feeble yellowish red if used on them directly. In the presence of aluminium compounds, intense red colors are formed; bluish violet in the presence of iron; and brownish violet in the presence of chromium. It has been used as stain for nervous tissue, but its chief present use is as an indicator.

Alizarin Carmine. See alizarin red S.

Alizarin No. 6. See purpurin.

Alizarin Purpurin. See purpurin.

Alizarin Red, Water Sol. See alizarin red S.

Alizarin Red S. (Syn. *Alizarin red, water sol. Alizarin carmine. Alizarin sulphate.*) A strongly acid dye, sodium alizarin sulphonate. It has been used by Benda for staining chromatin in preparations in which the mitochondria are stained with crystal violet. It has also been used as a vital stain for nervous tissue in small invertebrates.

Alizarin Sulphate. See alizarin red S.

Amaranth. (Syn. *Naphthol red. Fast red. Bordeaux. Bordeaux* SF. *Victoria rubin. Azo rubin. Wool red.*) A strongly acid red dye of the azo series, having little value as a protoplasm stain. It has been employed for staining axis cylinders, but is not one of the commonly used stains.

Amethyst Violet. (Syn. *Heliotrope* B. *Iris violet.*) A basic dye, tetraethyl pheno-safranin. It is seldom employed for biological staining, but has been recommended by Ehrlich and Lazarus in certain triple staining procedures.

Aniline Blue, Alc. Sol. See spirit blue.

* Conn, H. J. et. al. Biological Stains. Geneva, N. Y., 1925.

† Krause, Enzyklopädie der Mikroskopischen Technik. Ed. 3. Berl., 1926, 1927.

‡ Lee, A. B. The Microtomist's Vade-Mecum. Ed. 9. London, 1928.

§ Mallory, F. B. and Wright, J. H. Pathological Technic. Ed. 8. Phila., 1924.

Aniline Blue, Water Sol. (*Syn. China blue. Soluble blue 3M or 2R. Marine blue. Cotton blue. Water blue. Berlin blue.*) A strongly acid dye of the triphenyl methane series, derived from spirit blue by sulphonation; it is always a mixture, one of the components of which is methyl blue (q. v.). Unfortunately it is a dye whose exact composition cannot be controlled by the present methods of manufacture; it is therefore impossible to be sure of the identity of any two batches, even though made by the same manufacturer. For this reason it is a rather unsatisfactory dye to employ in any delicate staining procedure.

It is a widely used histological stain, having valuable properties as a counterstain. It is also employed as an indicator, but is much less satisfactory for the purpose than the sulphonephthaleins. The best known procedure at present for which it is required is the Mallory connective tissue stain, in which it is combined with orange G and acid fuchsin.

Aniline Red. See basic fuchsin.

Archelline 2B. See Bordeaux red.

Auramin. (Syn. *Canary yellow. Pyoktaninum aureum. Pyoktanin yellow.*) A basic dye of the diphenyl methane series, the only dye of the group concerning which references have been found in the literature on microscopic technique. Its chief use is as a drug, but it has been occasionally employed by the microscopist for some special procedure (p. 133).

Aurantia. (Syn. *Imperial yellow.*) An acid dye of the nitro series. It is obsolete as a textile dye and is almost unknown as a biological stain. It is called for, however, in combination with toluidine blue and acid fuchsin in the Champy-Kull technique for demonstrating certain cell constituents (mitochondria, etc.).

Aurin. See rosolic acid.

Aurin R. See corallin red.

Azidine Blue 3B. See trypan blue.

Azo-bordeaux. See Bordeaux red.

Azo-rubin. See amaranth.

Azure I. See methylene azure.

Azure II. See Giemsa stain.

Azure-II-eosin. See Giemsa stain.

Azure A. A lower homologue of methylene blue (a dimethyl instead of the tetramethyl derivative of thionin), a constituent of "azure I" as used in the Giemsa blood stain. At present it is prepared only by the oxidation of methylene blue, and has no commercial use.

It is called for in the latest formula recommended for the tetrachrome blood stain of MacNeal. It proves also of considerable value as a nuclear stain for sections of fixed tissue, especially for delicate procedures where a dye of more definite composition than methylene blue must be employed.

Azure B. This basic dye is the trimethyl derivative of thionin and therefore stands between methylene blue and azure A in composition and

properties. It is prepared, like azure A, from methylene blue by oxidation and has only recently been obtained in fairly pure form. It is a constituent of "azure I" and is therefore employed in making the Giemsa blood stain. Whether it has any special value as a stain has not yet been determined.

Azure C. A basic dye, mono-methyl thionin, prepared at present only by oxidation of methylene blue and not widely available. In composition and properties it stands between thionin and azure A. It has been recommended lately as a nuclear stain for tissues, in combination with eosin and orange II.

Basic Fuchsin. See fuchsin, basic.

Basic Rubin. See para-fuchsin.

Benzamine Blue 3B. See trypan blue.

Benzo Blue. See trypan blue.

Benzopurpurin 4B. (Syn. *Cotton red 4B. Dianil red 4C. Diamin red 4B. Sultan 4B. Direct red 4B.*) An acid dye of the azo series that has been used for vital staining, and has occasionally been employed as a protoplasm stain, especially in contrast to hematoxylin.

Berlin Blue. See aniline blue, w.s.

Biebrich Scarlet, Water Sol. (Syn. *Croceine scarlet, Scarlet B.* or *EC. Ponceau B. Double scarlet.*) An acid dye, a sulphonated Sudan III. Unlike the latter, it is water soluble and is not, therefore, a fat stain. It has occasionally been used as a protoplasm stain in contrast to methylene blue of hematoxylin.

Bismarck Brown G, R and GOOO. See Bismarck brown Y.

Bismarck Brown Y. (Syn. *Vesuvin. Phenylene brown. Manchester brown. Excelsior brown. Leather brown.*) A weakly acid dye of the azo series, formerly quite extensively used as a contrast stain, but now replaced to some extent by other counterstains. It is still used, however, as a mucin stain and is good for vital staining and for staining in bulk. It is employed in staining cellulose walls of plants in contrast to hematoxylin and occasionally for staining bacteria in contrast to gentian violet in the Gram technique. It produces a good stain for photomicrographic purposes and was extensively used by Foot and Strobell for staining smears of Hemipteran germ cells.

Blood Stains. See Giemsa stain, Jenner stain, Romanovsky stain, Tetrachrome stain, and Wright stain.

Bordeaux. See amaranth.

Bordeaux B, BL, G, and R. See Bordeaux red.

Bordeaux Red. (Syn. *Fast red B* or *P. Cerasin. Archelline 2B. Azo-bordeaux. Acid Bordeaux.*) An acid dye of the azo series, employed as a cytoplasmic stain, particularly when Heidenhain's hematoxylin is to be used immediately afterward as a nuclear stain. Also used in combination

with thionin and methyl green, for staining sections, especially of spleen, testis, and liver.

Bordeaux SF. See amaranth.

Brazilein.

Brilliant Blue C. See brilliant cresyl blue.

Brilliant Congo Red. See vital red.

Brilliant Cresyl Blue. (*Cresyl blue 2RN or BBS; Brilliant blue* C.) A basic dye of the oxazin group, valuable for certain special work on account of its highly metachromatic properties. Its chief biological use is for staining blood to demonstrate the platelets and reticulated corpuscles.

Brilliant cresyl blue is a difficult dye to manufacture and as there is no commercial demand for it, it is expensive. It proves quite a problem from the standpoint of standardization; and although considerably studied of recent years, it is not believed that all batches on the market are yet entirely reliable.

Brilliant Green. (Syn. *Ethyl green. Malachite green G.*) A basic dye of the triphenyl methane series. It has little if any use in microscopic technique; but is widely employed by the bacteriologist as a constituent of special culture media, as in the isolation of the typhoid organism and in the search for the colon organism in water. In these media it is used for its selective inhibitory properties on certain microorganisms.

Brilliant Pink. See rhodamine B.

Brom Chlor Phenol Blue. An acid dye of the sulphonephthalein series, changing from yellow to blue through the H-ion range between 3.0 and 4.6, and hence having use as an indicator. May also be used as an indicator of reaction in vital staining.

Brom Cresol Green. An acid dye of the sulphonephthalein series, used as an indicator, having a sensitive range between pH 3.8 and 5.4. This range is close to that of methyl red, and as the dye is not subject to reduction and fading of its color, it is often preferred to methyl red. Can be employed in microscopic work as indicator of reaction when used for vital staining.

Brom Cresol Purple. An acid dye of the sulphonephthalein series, much used as an indicator of reaction, having its sensitive range a little to the acid side of neutrality, namely between pH 5.2 and 6.8. For certain accurate chemical work it is undesirable because it is blue in reflected light but purple in transmitted light (in such cases substitute brom phenol red); but this dichromatism does not interfere with most of its biological uses. In vital staining, it also serves as an indicator of reaction.

Brom Phenol Blue. An acid dye of the sulphonephthalein series, employed as an indicator, with a sensitive range from pH 3.0 to 4.6. In vital staining it finds employment as an indicator of reaction.

Brom Phenol Red. An acid dye of the sulphonephthalein series, having indicator properties, with a sensitive range between pH 5.2 and 6.8. Can

be used as an indicator of reaction in vital staining. For accurate chemical work it is recommended in place of brom cresol purple, because it is free from the dichromatism which is sometimes troublesome in the latter.

Brom Thymol Blue. An acid dye of the sulphonephthalein series, one of the most useful indicators of reaction, as its sensitive range includes the neutral point, covering pH 6.0 to 7.6. In vital staining it also serves as an indicator of reaction.

Caesar Red. See eosin, bluish.

Canary Yellow. See auramin.

Carmine. Not an aniline dye (p. 435). (*Syn. Indigotine IA.*)

Cerasin. See Bordeaux red.

Cerasin Red. See Sudan III.

China Blue. See aniline blue, w.s.

Chlor Cresol Green. An acid dye of the sulphonephthalein series, having indicator properties, with a sensitive range between pH 4.0 and 5.6. May be used as an indicator of reaction in vital staining. Is frequently to be substituted for methyl red, when the unstable nature of the latter is objectionable.

Chlor Phenol Red. An acid dye of the sulphonephthalein series employed as an indicator, with a sensitive range between pH 4.8 and 6.4. Can occasionally be substituted for methyl red, where the unstable nature of the latter renders it undesirable. May also find employment in vital staining, where it can serve as an indicator within the tissue.

Chlorazol Blue 3B. See trypan blue.

Chrom Violet. An acid dye of the rosalic acid series.

Congo. See Congo red.

Congo Blue 3B. See trypan blue.

Congo Red. (Syn. *Congo. Cotton red. Direct red.*) An acid dye of the azo group, best known to the biologist as an indicator. It also has certain value as a histological stain, as for demonstrating axis cylinders in nervous tissue, and mucin in plants, as well as serving for a general background stain in contrast to hematoxylin, and other nuclear dyes.

Corralin Yellow. The sodium salt of rosalic acid.

Cotton Blue. See methyl blue.

Cotton Red. See Congo red.

Cotton Red 4B. See benzopurpurin 4B.

Cresol Red. An acid dye of the sulphonephthalein series, used as an indicator, having a sensitive range between pH 7.2 and 8.8. In vital staining it also serves as an indicator of reaction.

Cresolphthalein. A weakly acid dye of the xanthene series, very closely related to the much better known phenolphthalein. Like the latter, it is employed as an indicator, and has a very similar range (pH 8.3 to 10.0). For some purposes it is preferable to phenolphthalein.

Cresyl Blue 2RN or BBS. See brilliant cresyl blue.

Cresylecht Violet. See cresyl violet.

Cresyl Violet. (Syn. *Cresylecht violet*, i. e., Cresyl fast violet.) A basic dye of the oxazin group. It is not a textile dye, and is not widely used as a stain; but finds some employment because of its strongly metachromatic properties. According to Ehrlich, it stains nuclei violet, plasma blue, amyloid, mucin, and mast cell granules red. It is valuable for making permanent preparations of nervous tissue. It has recently been employed for staining sections of fresh tumor tissue in diagnostic and autopsy work.

At least two different dyes have been used in the past by biologists under this name and some confusion has resulted. There is no question as to which is correctly termed cresyl violet, but it is doubtful whether in any given instance this is the dye actually used by the author of some technique calling for a stain so named. The matter is still under investigation.

Croceine Scarlet. See Biebrich scarlet, water sol.

Crystal Violet. (Syn. *Violet C, G* or *7B. Hexamethyl violet. Methyl violet 10B. Gentian violet.*) A basic dye of the triphenyl methane group—hexamethyl pararosaniline. It is a constituent of all the bluer shades of methyl violet and gentian violet; and apparently the most important constituent of pre-war gentian violet for the purposes for which the microscopist employed the latter (see gentian violet). Crystal violet has been definitely specified in certain procedures, such as Benda's crystal-violet-alizarin method for staining chondriosomes, and in a recently proposed stain (Jackson, 1926) in combination with erythrosin for staining lightly lignified walls in plants. Besides such special procedures as this, it is now highly recommended in nearly all procedures calling for gentian violet, as crystal violet is a definite chemical compound and therefore more constant in composition than gentian violet. The only exception to this is that in certain procedures crystal violet is slightly too blue for the proper contrast; in such instances one of the methyl violets (2B or redder) is to be recommended (see methyl violet).

Dahlia. See Hoffman violet.

Dahlia B. See methyl violet.

Diamine Red 4B. See benzopurpurin 4B.

Diamond Fuchsin. See basic fuchsin.

Diamond Green. See malachite green.

Dianil Blue H3G. See trypan blue.

Dianil Red 4C. See benzopurpurin 4B.

Dianthin B. See erythrosin, bluish.

Dianthin G. See eosin, bluish.

Diazin Green. See Janus green B.

Direct Red. See Congo red.

Direct Red 4B. See benzopurpurin 4B.

Double Green. See methyl green.

Double Scarlet. See Biebrich scarlet, water sol.

Emerald Green. See malachite green.

Eosin, Alcohol Sol. See methyl eosin and ethyl eosin.

Eosin, Bluish. (Syn. *Eosin, BN, B, BW,* or *DKV. Safrosin. Eosin scarlet B* or *BB. Scarlet J's JJ's* or *V. Nopalin G. Caesar red.*) An acid dye of the xanthene series dinitro-dibrom fluorescein. It has occasionally been called for as a counterstain in some histological procedure, but is not in general very valuable. Ordinarily, if a shade deeper than eosin Y is desired, better results can be obtained with erythrosin, phloxine or rose bengal than with eosin B. One company, in fact, puts on the market a product labelled "eosin, bluish blend," (formerly sold as eosin bluish) which is a mixture of eosin Y with some one of the dyes just named; it is very satisfactory for certain staining procedures, but must not be confused with true eosin B.

Eosin BN, B, BW, and **DKV.** See eosin, bluish.

Eosin J. See erythrosin, bluish.

Eosin S. See ethyl eosin.

Eosin Scarlet B and **BB.** See eosin bluish.

Eosin W or **WS.** See eosin Y.

Eosin Y. (Syn. *Water sol. eosin. Eosin W* or *WS.*) An acid dye of the xanthene group—tetrabrom fluorescein. There are several other eosins besides eosin Y, all derivatives of fluorescein, but this grade (i. e. yellowish eosin) is ordinarily referred to when eosin alone is specified.

Eosin Y is probably the most commonly used of the red acid dyes. It is one of the most valuable cytoplasm stains known and is employed in a great variety of procedures. It is most frequently used as a counterstain for hematoxylin and the green or blue basic dyes; also by Mann, mixed with methyl blue, as a tissue stain. One of the uses for which it is at present in greatest demand is in the various blood stains, in which it is combined with methylene blue or with some oxidation product of the latter (see Giemsa stain, Jenner stain, Romanovsky stain, tetrachrome stain, Wright stain).

Erythrosin B. See erythrosin bluish.

Erythrosin BB. See phloxine.

Erythrosin, Bluish. (Syn. *Erythrosin B. Pyrosin B. Eosin J. Iodoeosin B. Dianthin B.*) An acid dye of the xanthene group—tetra-iodo fluorescein. This is probably the dye that has been used in the past in instances where "erythrosin" is specified. No procedures are definitely known, in fact, where the yellowish erythrosin is more satisfactory than the bluish type; and as the former is an obsolete dye, while the latter is a recognized food color, the bluish type is the one now ordinarily obtainable.

Erythrosin is used somewhat as an indicator; and has fairly extensive employment as an histological stain, although not so widely used as eosin Y. Its value for histological purposes is as a red counterstain not quite so yellow and not quite so strongly acid in character as eosin Y. These two properties make it useful in some procedures where eosin Y is not

wholly satisfactory. It has recently been employed as a stain for bacteria in soil.

Erythrosin R or **G.** See erythrosin, yellowish.

Erythrosin, Yellowish. (Syn. *Erythrosin R* or *G. Pyrosin J. Dianthin* G. *Iodo-eosin* G.) An acid dye of the xanthene group—di-iodo fluorescein. It may have been used in the past for some procedures in which simply "erythrosin" has been named. The bluish grade is now ordinarily employed, however.

Ethyl Eosin. (Syn. *Eosin, alcohol sol. Eosin S.*) An acid dye of the xanthene group, the ethyl ester of eosin Y. This dye has ordinarily been sold in the past as alcohol soluble eosin; but the term ethyl eosin is to be preferred as there are other alcohol soluble eosins, particularly the one known as methyl eosin. Apparently the ethyl compound is the one that was available before the war. Since the war there has been some confusion in the matter.

It is a very valuable counterstain after Delafield's hematoxylin in general histological work. It is specially called for in contrast to methylene blue in searching for Negri bodies in the nervous tissue of animals suspected of having had rabies.

Ethyl Green. See brilliant green.

Excelsior Brown. See Bismarck brown Y.

Fast Acid Green N. See light green SF, yellowish.

Fast Blue B. See naphthol blue.

Fast Blue 3R. See naphthol blue.

Fast Red. See amaranth.

Fast Red B or **P.** See Bordeaux red.

Fast Yellow. An acid dye of the azo group closely related to methyl orange, rarely employed as a biological stain, although occasionally called for in certain special procedures.

Fettponceau. See Sudan IV.

Fluorescein. (Syn. *Uranin.*) An acid dye, the simplest member of the fluorane group and the mother substance of the eosins. It is of low tinctorial power, but has a very marked yellow fluorescence which is detectable even in extremely great dilution. The dye has no apparent value as a biological stain.

Fuchsin, Acid. (Syn. *Fuchsin S, SN, SS, ST,* or *S III. Acid magenta. Acid rubin.*) An acid dye (or group of dyes) of the trimethyl methane series, derived from basic fuchsin by sulphonation. As there are four primary basic fuchsins theoretically possible, and as each one of them may yield a mono-, di- or trisulphonic acid, an extremely great variety of different acid fuchsins may occur, and its manufacture is very difficult to control so as to produce a uniform product. Recent work by Scanlan, Holmes and French,* however,

* Scanlan, Holmes, and French, *Stain Technology.* 1927, ii, 50.

shows that to a certain extent such control is possible, and the future may see a more uniform product supplied to microscopists.

Acid fuchsin is one of the most widely used cytoplasm stains. It has also been called for in many special procedures, such as the Van Gieson (p. 289) connective tissue stain, in which it is used with picric acid after hematoxylin to differentiate smooth muscle from connective tissue; the Ehrlich-Biondi stain, in which with methyl green and orange G it is employed in histology and for staining blood smears; the Ehrlich tri-acid stain for blood, in which it is combined with orange G and methyl green; the Pianese stain for cancer tissue (with malachite green and martius yellow), now mostly used in studying infected vascular plants; as a constituent of the Mallory aniline blue connective tissue stain; and with methyl green as a stain for mitochondria.

Fuchsin, Basic. (Syn. *Diamond fuchsin. Magenta. Rubin. Aniline red.*) A basic dye (or group of dyes) of the triphenyl methane group. There are four primary basic fuchsins theoretically possible, according to the number of methyl groups introduced into the benzene rings. If no such methyl group is present the compound is known as pararosanilin; if one, it is known as rosanilin; if three, as new fuchsin. All three of these compounds are known in fairly pure form. The compound intermediate between rosaniline and new fuchsin, although possible and undoubtedly present in some of the basic fuchsins, is not obtained pure without special methods of preparation, and is not a recognized dye. Ordinary basic fuchsins are mixtures of pararosanilin and rosanilin.

Basic fuchsin is one of the most powerful nuclear dyes, almost too powerful for delicate histological work, on account of its tendency to overstain. It is employed for mucin and elastic tissue; but by far its most important microscopic use is as a bacterial stain, particularly in the Ziehl-Neelson method for differentiating the tubercle organism and thus diagnosing tuberculosis. The bacteriologist also uses it, in decolorized form, in the Endo medium as an indicator to distinguish between the typhoid and colon organisms, thanks to the property of the latter of restoring the red color if growing in the presence of lactose.

Considerable work has been done recently to learn which of the types of basic fuchsin are best adapted to the various purposes. No positive conclusions have yet been reached, but certain general statements can be made. Almost any dye of the group will do as a simple bacterial stain; but for the Ziehl-Neelson technique, rosanilin or even new fuchsin seems to prove better than pararosanilin. For the Endo medium, however, better results are obtained with pararosanilin, or at least with a mixture of pararosanilin and rosanilin. Few of the basic fuchsins on the market are satisfactory for both purposes, although certain lots work as well for one technique as for the other. An investigation has been made to learn the

actual chemical nature of these generally satisfactory lots; but as yet it has not been discovered just how they differ from the others.

Fuchsin NB. See new fuchsin.

Fuchsin S, SN, SS, ST, or **S III.** See acid fuchsin.

Gentian Blue. See spirit blue.

Gentian Violet. This is not a textile dye, and is not recognized in the regular dye industry. Under this name, Grübler sold to biologists a mixture of one of the higher methyl violets with crystal violet. It may therefore be regarded as a very highly methylated methyl violet. Soon after the war it was found that some dealers in the United States were selling crystal violet under the name of gentian violet, and as this was found to give better results than most of the methyl violets, the practice has been recognized by the adoption of the following definition of gentian violet by the Commission on Standardization of Biological Stains:

"Gentian violet . . . must be either pentamethyl or hexamethyl pararosanilin, or else a mixture of methylated pararosanilins composed primarily of the compounds just named and having a shade at least as deep as that recognized in the trade as methyl violet 2B."

The Commission is at present recognizing two grades of gentian violet: one gentian violet bluish, which is primarily or entirely crystal violet; the other gentian violet reddish, which is apparently a mixture, one of the deeper methyl violets.

This stain is of chief value to the biologist as a nuclear or chromatin stain, having many histological and cytological applications, the one for which it is most commonly employed at present being the Flemming triple stain (with orange G and safranin). It is also used for various histological procedures. It is an extremely valuable bacterial stain, especially in the Gram technique, with its various modifications. Recently it has been further employed for its inhibiting effect on certain bacteria and has been suggested as a therapeutic agent.

For the Gram stain (as well as for staining bacteria in general) the bluish grade or crystal violet proves most satisfactory; this grade is likewise to be preferred in most histological and cytological work, including the Flemming technique. In certain variations of the Flemming stain, however, some microscopists seem to get better results with the reddish grade. It has also been stated that crystal violet alone does not have such pronounced inhibiting action on bacteria as do some of the mixtures that have been sold as gentian violet.

Giemsa Stain. (Syn. *Azure-II-eosin.*) A so-called "neutral stain," used in staining blood; a compound of eosin with one of the derivatives of methylene blue, known as "azure II." Azure II is a mixture of azure I (see methylene azure) and methylene blue in equal parts. In the Giemsa stain this mixture is compounded with eosin, and the eosinates which are formed, being practically insoluble in water, precipitate out. This preci-

pitate is the Giemsa stain, and can be used in alcoholic solution, as are the Wright stain and tetrachrome stain.

Gold Orange. See orange II.

Gray R, B, BB. See nigrosin, water sol.

Hematein.

Helianthin. See methyl orange.

Heliotrope B. See amethyst violet.

Helvetia Blue. See methyl blue.

Hexamethyl Violet. See crystal violet.

Hoffman Green. See iodine green.

Hoffman Violet. (Syn. *Dablia. Iodine violet. Red violet. Violet R, RR,* or *4RN.*) A basic dye of the triphenyl-methane series, closely related to methyl violet, but having ethylated instead of methylated amino groups. Like methyl violet it may vary considerably in shade, as it is a mixture of various homologues of the series.

The dye has been used occasionally in histological work, and has been specially recommended for staining mast cells and amyloid. It has considerable metachromatic effect.

Imperial Yellow. See aurantia.

Indian Blue 3RD. See naphthol blue.

Indigo. (Syn. *Indigo blue.*) A well-known blue dye formerly obtained from certain species of plants, but now ordinarily manufactured synthetically. It was formerly employed to some extent as a biological stain: but has little such use at the present time.

Indigo Blue. See indigo.

Indigo Carmine. (Syn. *Indigotine IA.*) A blue dye closely related to indigo, which is sometimes used as a cytoplasm stain in contrast to carmine.

Indigotine IA. See indigo carmine.

Indin Blue 2RD. See naphthol blue.

Indulin Black. See nigrosin, water sol.

Iodo-eosin B. See erythrosin, bluish.

Iodo-eosin G. See eosin, bluish.

Iodine Green. (Syn. *Hoffman green.*) A basic dye of the triphenyl-methane series, closely related to methyl green, differing from the latter in being a rosanilin derivative while the latter is supposed to be derived primarily from pararosanilin. It is a nuclear or chromatin stain having selective properties that make it of value in certain special procedures. It has been used for staining nervous tissue in combination with acid fuchsin and picric acid, and as a blood stain in combination with acid fuchsin; with basic fuchsin it has been employed for staining chromatin in plant tissue, and with acid fuchsin for lignified xylem. It is also employed for staining mucin and amyloid, having the property of giving the latter a red instead of a green color.

Iodine Violet. See Hoffman violet.

Iris Violet. See amethyst violet.

Isorubin. See new fuchsin.

Janus Green B. (Syn. *Diazin green.*) A basic dye related both to the safranins and to the azo dyes. It is best known to the biologist for its use in demonstrating chondriosomes, stained **intra vitam.** It has also been employed with neutral red for staining sections of embryos.

Jenner Stain. One of the first compounds of eosin and methylene blue used for staining blood. It is still employed to some extent, although for most purposes it gives much less satisfactory results than do the Giemsa, the Wright or the tetrachrome stains (q. v.). Jenner's stain is merely the eosinate of methylene blue, precipitated from aqueous solution and redissolved in methyl alcohol. As polychrome methylene blue is not used in preparing it, the stain does not give the many colored effects obtained with any one of the other three blood stains just mentioned.

Lauth's Violet. See thionin.

Leather Brown. See Bismarck brown Y.

Light Green SF, Yellowish. (Syn. *Light green 2G, 3G, 4G,* or *2GN. Acid green. Fast acid green N.*) An acid dye of the triphenyl-methane series. It is a valuable cytoplasm stain often employed for staining tissues in contrast to iron hematoxylin, although it fades badly if exposed to bright light. It has also been used in contrast to safranin as a cytoplasm stain for spermatozoa, and as a cytoplasm and cellulose stain in plant histology.

Care must be taken not to confuse this dye with other light greens (such as malachite green and methyl green). If the grade "SF yellowish" is not specified in ordering there is danger of obtaining some other dye than the one desired.

Light Green 2G, 3G, 4G, or **2GN.** See light green SF, yellowish.

Light Green N. See malachite green.

Litmus. A natural dye obtained from certain lichens. It is used chiefly as an indicator; and at present much less even for that purpose than formerly.

Lyons Blue. See spirit blue.

Magdala Red. (Syn. *Naphthaline red. Naphthaline pink. Naphthylamine pink. Sudan red.*) A basic dye of the safranin group (a naphtho-safranin). True Magdala red is very expensive, and was sold before the war under the name "Magdala rot, echt." A cheaper "Magdala red," without qualifying designation, was also put on the market by Grübler before the war, which proves to have been (in some instances, at least) a fluorescein derivative (phloxine or erythrosin) rather than a safranin. It is possible to obtain a fluorescein dye of exactly the shade of Magdala red, indistinguishable from it even by spectrophotometric analysis, but it has acid instead of basic properties. Such seems to have been the nature of the cheaper "Magdala red" available before the war. At least one instance is known in which this

has led to confusion. Chamberlain* has employed "Magdala red" with aniline blue in staining algae; but finds that true magdala red is unsatisfactory in the technique, while phloxine gives him more dependable results than obtained with the so-called "Magdala red" which he formerly used. Magdala red has also been employed occasionally in animal histology, either as a nuclear or an elastin stain; for these purposes probably the true dye was used.

Magenta. See basic fuchsin.

Malachite Green. (Syn. *Emerald green. New Victoria green. Diamond green. Solid green. Light green* N.) A weakly basic dye of the triphenyl methane series. It was formerly employed for various histological purposes, but today has largely been replaced by methyl green. It is still used by botanists (together with acid fuchsin and martius yellow) for staining host tissue in plants infected with fungi.

Although this dye is also known as light green N, it is not the dye which the microscopist wants in procedures calling for light green. Light green SF yellowish is the dye ordinarily to be employed in such procedures, but when light green, without qualifying designation, is ordered, the dye manufacturer sometimes furnishes malachite green.

Malachite Green G. See brilliant green.

Manchester Brown. See Bismarck brown Y.

Manchester Yellow. See Martius yellow.

Mandarin G. See orange II.

Marine Blue. See aniline blue, W.S.

Martius Yellow. (Syn. *Manchester yellow. Naphthol yellow.*) An acid dye of the nitro series, quite closely related to picric acid. It was used by Pianese in combination with malachite green and acid fuchsin for studying cancer tissue; the same technique is now applied by botanists to the study of sections of plants infected by fungi. The dye is also employed in preparing certain light filters for photomicrography.

Meldola's Blue. See naphthol blue.

Methyl Blue. (Syn. *Cotton blue. Helvetia blue.*) An acid dye of the triphenyl methane series, it is one of the components of aniline blue, water soluble (q. v.). It is largely employed in the Mann (p. 402) technique (with eosin) for staining nerve cells. It is a good counterstain, for contrast to a red nuclear stain such as safranin or carmine; picric acid is sometimes used with it when it is thus employed.

Meta-cresol Purple. An acid dye of the sulphonephthalein series, used for its indicator properties, changing from red to yellow through the range between pH 1.2 and 2.8, and from yellow to purple between pH 7.4 and 9.0. Can be employed in microscopic work as an indicator of reaction when used as a vital stain.

* Chamberlain, C. J. *Stain Technology*, 1927, ii, 91.

Methyl Eosin. (Syn. *Eosin, alcohol sol.*). An acid dye of the xanthene group, the methyl ester of eosin Y. It may sometimes have been employed where eosin, alcohol soluble, has been specified, although for this purpose the dye most frequently used has probably been ethyl eosin (q. v.).

Methyl Green. (Syn. *Double green. Light green.*) A basic dye of the triphenylmethane series, closely related to iodine green, derived from crystal violet by the introduction of a seventh methyl group through the action of methyl chloride or iodide. It is readily decomposed and this added methyl group driven off. Thus some of the violet dye from which it is derived is almost sure to be present; and this fact partly, at least, explains the metachromatic action of methyl green.

Methyl green is an extremely valuable nuclear stain in histology and chromatin stain in cytology, and is used with various counterstains especially acid fuchsin. Ehrlich has combined it to form a "neutral stain" with orange G and acid fuchsin, and employs the compound as a blood stain. Botanists find it valuable for lignified xylem, with acid fuchsin for contrast. It is much used today mixed with pyronin in the Pappenheim stain, employed for staining the gonococcus and mast cells. It is a very valuable chromatin stain for protozoa.

Following the war, methyl green was one of the most difficult stains to obtain in satisfactory quality. The problem seems to have been solved completely now, however, and for some time no complaints of American methyl greens have been heard.

Methyl Orange. (Syn. *Orange III. Helianthin. Gold orange. Tropeolin D.*) A weakly acid dye of the azo series, chiefly employed as an indicator. It is occasionally used in histology, however, as a counterstain and in one or two special procedures.

Methyl Red. An old and well known indicator, having a sensitive range between pH 4.4 and 6.0. Where a quick reading is desired it is excellent but the color fades rapidly. Hence it is not suitable for the preparation of color standards, nor for work where it is desired to study H-ion changes in a solution to which an indicator is added. For such purposes it should be replaced by one of the sulphonephthalein indicators—either brom cresol green or chlor cresol green if the acid half of its range is under consideration, or chlor phenol red in the case of the alkaline end of the range.

Methyl-violet. (Syn. *Dahlia B. Paris violet. Pyoktanin blue. Gentian violet.*) A basic dye of the triphenyl methane series. (See also gentian violet and crystal violet.) It is a mixture of tetramethyl, pentamethyl, and hexamethyl rosanilins or pararosanilins. The hexamethyl compound alone is known as crystal violet. The greater the degree of methylation the less red and the more blue is its shade; this leads to various shade designations such as 2R, R, B, 2B, 5B, 6B, etc. (R standing for red, B for blue). Each designation, such as *methyl violet 2B,* indicates a fairly definite shade, but not a

definite chemical composition, as the same shade may be obtained by different mixtures of compounds in this group.

Methyl violet, sometimes with and sometimes without shade designation, has been specified in various procedures as a nuclear stain and sometimes as a bacterial stain in the Gram technique. For some of these procedures, as good (or better) results can be obtained with crystal violet alone as with one of the indefinite mixtures known as methyl violet; in such cases, crystal violet should be used, and specified for the technique in question, because it is a definitely known chemical compound. In some instances, however, crystal violet is not sufficiently reddish in shade to give the proper contrast; then a methyl violet should be used. For such purposes the grade 2B seems fairly satisfactory; it is distinctly redder than crystal violet and yet not so red but that it will show contrast to a red counter-stain.

Methyl-violet 10B. See crystal violet.

Methylene Azure. (Syn. *Azure I.*) A name given to an oxidation product of methylene blue, consisting partly of azure A and partly of azure B. It is called for in the Giemsa stain for blood (q. v.) and was originally specified in the tetrachrome stain. More recent formulae of the latter, however, call for azure A instead of the mixture formerly known as azure I (see tetrachrome stain). Apparently in most instances where methylene azure is called for, azure A can be used to best advantage.

Methylene Blue. (Syn. *Swiss blue.*) A basic dye of the thiazin series, tetramethyl thionin. To the pathologist and bacteriologist methylene blue is perhaps the most valuable of all the stains, while the zoologist also finds many uses for it. It is used, first, as a nuclear stain in histology; second, as a bacterial stain, notably in milk work and in the diagnosis of diphtheria; third, in the vital staining of nervous tissue and fourth, in combination with eosin in the blood stains.

Its greatest value along these lines is that it is a strong basic dye without great tendency to overstain, and that it has very pronounced metachromatic properties. These metachromatic properties are especially likely to develop in methylene blue solutions on standing, and such a solution is known as "polychrome methylene blue." Its formation is hastened by boiling with alkali. What actually happens is that the methylene blue is partly oxidized into its lower homologues, methylene violet and the azures (q. v.). In preparing Wright's stain, methylene blue is polychromed before combining with eosin.

The ease with which methylene blue oxidizes into these lower homologues makes it almost impossible to secure a pure methylene blue. Practically all, if not all, commercial methylene blues are partly polychromed, and it has been claimed that the most valuable properties of methylene blue are the oxidation products thus produced. It appears, in fact, that pure methylene blue is a relatively poor nuclear stain, with very little

metachromasy, while the lower homologues are successively better nuclear stains and more highly metachromatic.

Before the war some of the methylene blue sold to biologists was in the form of the zinc double salt. This form is quite insoluble in alcohol and is a comparatively poor stain. For all staining purposes investigated, better results can be obtained with the medicinal grade of methylene blue, which according to the U.S.P. requirements must be zinc-free. It is the form in which methylene blue is now ordinarily furnished to biologists in America.

Methylene Blue NN. See new methylene blue N.

Methylene Blue O. See toluidine blue O.

Methylene Green. A nitro derivative of methylene blue. It is occasionally used as a substitute for methyl green, especially by botanists in the case of wood and fixed chromatin; it gives good results in combination with eosin.

Methylene Violet. Bernthsen. This is not a textile dye, and must be distinguished from methylene violet, RRA or 3RA which is better known commercially. It is one of the oxidation products of methylene blue. It plays an important part in the nuclear and granule staining of polychrome methylene blue. A definite quantity of this dye is mixed with methylene blue, azure A, and eosin in the tetrachrome blood stain of MacNeal, p. 458.

Naphthaline Pink. See Magdala red.

Naphthaline R. See naphthol blue.

Naphthaline Red. See Magdala red.

Naphthamine Blue, 3BX. See trypan blue.

Naphthol Blue. (Syn. *New blue B. Fast blue 3R. Phenylene blue. Meldola's blue. Indin blue 2RD.*) A basic dye of the oxazin group, which has been occasionally employed as a vital stain.

Naphthol Red. See amaranth.

Naphthol Yellow. See Martius yellow.

Naphthylamine Pink. See Magdala red.

Narcein. An acid dye of the azo series, closely related to orange II. It has very little use as a stain, but has been employed in combination with pyronin and methyl green or methylene blue to form a compound dye.

Neutral Red. A weakly basic dye of the azin series, ordinarily the chloride of toluylene red. It has recently been proposed to employ the iodide, however, as it is claimed to be more easily purified than the chloride.

Neutral red is best known as an indicator, but has special staining value where a weakly basic, non-toxic dye is called for. It has been employed as a vital nuclear stain; for the staining of fresh blood or pus in a moist chamber; for the vital staining of protozoa; for bringing out Nissl granules in nerve cells; and it has some use in general histological staining, especially for embryological tissue in combination with Janus green.

Neutral Violet. A weakly basic dye of the azin group, closely related to neutral red and having very similar properties. It has rarely been used in microscopic work.

New Blue B. See Naphthol blue.

New Fuchsin. (Syn. *Isorubin. Fuchsin NB.*) A basic dye of the triphenyl methane series. It is sometimes furnished as basic fuchsin, either alone or mixed with rosanilin and pararosanilin; it differs from rosanilin in that three methyl groups instead of one are attached to the benzene rings. It is seldom recommended as a stain, but basic fuchsins containing a large proportion of it have been found to be splendid stains for certain purposes. A new fuchsin, for instance, may prove better in the Ziehl-Neelson stain for the tubercle organism than the lower members of this group of dyes.

New Methylene Blue N. (Syn. *Methylene blue NN.*) A basic dye of the thiazin group, of somewhat greener shade than true methylene blue. It apparently was present in certain pre-war lots of methylene blue; and it has been found that in at least one instance results carried on with pre-war "methylene blue" can be duplicated with new methylene blue, but not with true methylene blue.

New Pink. See phloxine.

New Victoria Green. See malachite green.

Niagara Blue 3B. See trypan blue.

Night Blue. See spirit blue.

Nigrosin, Water Sol. (Syn. *Nigrosin W, WL,* etc. *Gray R, B, BB. Silver gray. Steel gray. Indulin black.*) A basic dye of poorly understood composition. It is apparently a mixture of an indulin (violet in color) with a yellow dye so as to make an almost black solution. It has been recommended for staining various kinds of tissue, either alone or in combination with other stains such as hematoxylin. It is employed in studying algae and fungi. One of its most striking uses is in the Dorner spore stain for bacteria, in which technique it has the property of withdrawing basic fuchsin from the vegetative portions of the cells but not from the spores. But as it itself does not stain the cells, they stand out colorless in a gray background, while the spores retain the fuchsin.

Nile Blue A. See Nile blue sulphate.

Nile Blue Sulphate. (Syn. *Nile blue A.*) A basic dye oft he oxazin series, closely related to brilliant cresyl blue. The use for which this dye is best known to the biologist is the Lorrain Smith fat stain. In this procedure, the dye is boiled with dilute sulphuric acid and is thus partly converted into a new dye of the class known as oxazones. This oxazone dye is red and is fat-soluble; while Nile blue itself is not fat-soluble, but combines readily with fatty acids. As a result, the technique serves to distinguish between the free fatty acids in histological material and the neutralized fats, the former staining blue, the latter red.

Nopalin G. See eosin, bluish.

Oil Red. See Sudan III.

Oil Red IV. See Sudan IV.

Oil Red O. (Syn. *Oil scarlet, Sudan II, X,* or AX.) A fat-soluble dye of the azo group. It has not until recently been employed for microscopic work; but has lately been recommended,* for staining fat in sections.

Oil Scarlet. See oil red O.

Orange II. (Syn. *Gold orange. Orange A, P* or *R. Acid orange. Orange extra. Mandarin G. Tropeolin* 000 *No.* 2.) An acid dye of the azo series. It has some value as a counterstain, having recently been recommended by French for use with azure C and eosin as a tissue stain.

Orange III. See methyl orange.

Orange A, P, or **R.** See orange II.

Orange Extra. See orange II.

Orange G. (Syn. *Wool orange 2G.*) An acid dye of the azo series. It is one of the most valuable histological counterstains, and is the orange dye most frequently used by histologists. It is employed with various nuclear stains, but special mention should be made of safranin and gentian violet (with which it is used in the Flemming triple stain), and of methyl green and acid fuchsin (with which it is combined in the technique of Ehrlich-Biondi-Heidenhain p. 135). In the Mallory connective tissue stain it is employed with two other acid dyes, aniline blue and acid fuchsin. A further use is Bensley's "neutral gentian," a combination of orange G with gentian violet for staining the islands of Langerhans.

Orange GG, GMP. See orange G.

Orcein. A natural dye, closely related to litmus, obtained from certain lichens. It is a weak acid, with a violet color. As a biological stain it seems to have been used chiefly by Unna (p. 297), who employs it for staining connective tissue, plasma fibrils in the epithelium, and for other purposes. Other histologists have used it, however, for certain special procedures, as for coloring elastic tissue.

Para-fuchsin. See pararosanilin.

Para-magenta. See pararosanilin.

Pararosanilin. (Syn. *Basic rubin, Para-fuchsin, Para-magenta.*) A basic dye of the triphenyl-methane series, the lowest member of the group known as basic fuchsins. See basic fuchsin.

Pararosolic Acid. An acid dye, a hydroxyl derivative of triphenyl methane. Generally present in commercial preparations of rosolic acid.

Paris Blue. See spirit blue.

Paris Violet. See methyl violet.

Phenolphthalein. A weak acid dye of the xanthene series, related to the fluorescein dyes (eosin, etc.). It is used primarily as an indicator, having its sensitive range between pH 8.3 and pH 10.0.

Phenol Red. An acid dye of the sulphonephthalein series, one of the longest known of the indicators of this group. Its range (pH 6.8 to 8.4) lies

* French, R. W. *Stain Technology.* 1926, i, 78.
Proescher, F. *Stain Technology.* 1927, ii, 60.

close to neutrality, and the indicator has proved valuable for some time in urine analysis. Under the microscope it also serves as an indicator of reaction when employed as a vital stain.

Phenylene Blue. See naphthol blue.

Phenylene Brown. See Bismarck brown Y.

Phloxine. (Syn. *Erythrosin BB. New pink.*) An acid dye of the xanthene series—dichlor or tetrachlor tetrabrom fluorescein. It is a distinctly red dye with little of the orange tinge noticeable in the lower homologues, eosin and erythrosin. It proves a good counterstain in contrast to certain blue dyes of the thiazin series. It works better than eosin in the "eosin-methylene-blue" stain of Mallory for sections of tissue. Under certain conditions it is a very good bacterial stain. Chamberlain (p. 167) finds that it gives much better results than magdala red in staining algae; and concludes that the "magdala red" which he used before the war must have contained phloxine or some closely related dye.

Picric Acid. A strongly acid dye of the nitro series, yellow in color. One of the most common uses of picric acid is in contrast to acid fuchsin in the Van Gieson connective tissue stain. It is also employed as a general cytoplasmic stain in contrast to various basic dyes. It has further application as a fixative.

Ponceau B. See Biebrich scarlet, water sol.

Ponceau 3B. See Sudan IV.

Purpurin. (Syn. *Alizarin No. 6. Alizarin purpurin.*) An acid dye of the oxyquinone group, closely related to alizarin. It is a little darker in shade than alizarin. It has been used as a nuclear stain for histological material, and for determining the presence of insoluble calcium salts in cell contents.

Pyoktanin Blue. See methyl violet.

Pyoktanin Yellow. See auramin.

Pyoktaninum Aurem. See auramin.

Pyronin B and G. Two very closely related dyes of the xanthene series. Pyronin G is tetra-methyl diamino xanthene, while pyronin B is the corresponding tetra-ethyl compound. Pyronin G was apparently the dye used before the war; but it is no longer available, and pyronin B seems to be a satisfactory substitute in histological work. It is most used in combination with methyl green (the Pappenheim stain) for staining various basophile elements such as mast cells and for staining the gonococcus in smears of pus. In the formula for the Pappenheim stain allowance must be made for the fact that the American pyronins are more concentrated than those used before the war; and a smaller amount in proportion to methyl green should be employed.

Pyrosin B. See erythrosin, bluish.

Pyrosin J. See erythrosin, yellowish.

Red Violet. See Hoffman violet.

Rhodamine B. (Syn. *Rhodamine O. Brilliant pink.*) A weakly basic dye of the xanthene series. It has been used in combination with osmic acid as a solution for simultaneous fixing and staining. It has also been employed for histological work in contrast to methylene blue or methyl green; and as a component of certain compound stains.

Rhodamine O. See rhodamine B.

Romanovsky Stain. The first compound of eosin and methylene blue proposed for staining blood. It was merely a mixture of the two dyes without effort to precipitate and remove the compound dye formed; and yet its author realized that some new dye was formed in the solution. The methylene blue solution was not intentionally polychromed, although Romanovsky discovered that old solutions gave best results.

Rosanilin. A basic dye of the triphenyl methane group, differing from pararosanilin in that a methyl group is attached to one of the benzene rings of the latter. It is a member of the group of compounds known as basic fuchsins. See basic fuchsin.

Rose Bengal. An acid dye of the xanthene series—dichlor or tetrachlor tetra-iodo fluorescein. It is even more distinctly red than phloxine, to which it is closely related; and often shows a slight violet cast in staining. As a result it is not such a good counterstain to the blue dyes as the lower members of the group. It has been employed as a bacterial stain, especially for staining bacteria in soil suspensions.

Rosolic Acid. (Syn. *Aurin.*) An acid dye, a hydroxyl derivative of triphenyl methane. Its chief use is as an indicator.

Rubin. See basic fuchsin.

Safranin O. A basic dye of the azin group. There are various safranins but the grade desired by biologists seems to be a certain mixture of dimethyl and trimethyl phenosafranins, having a definite shade of red not easily recognized by eye but characterized by having a light absorption curve with its maximum at or near 515μ. It is one of the most important nuclear and chromatin stains known. It is especially valuable to the plant histologist for bringing out lignified and cutinized tissues, and as a protein stain. The cytologist makes use of it in the Benda technique to stain chromatin in combination with light green as a contrast stain; and even more widely in the Flemming triple stain, in which it is employed to bring out the chromatin, with gentian violet and orange G for contrast. The bacteriologist uses it occasionally, especially as a counterstain in the Gram technique.

Safrosin. See eosin, bluish.

Scarlet B or **EC.** See Biebrich scarlet, water sol.

Scarlet G or **B.** See Sudan III.

Scarlet J, JJ or **V.** See eosin, bluish.

Scarlet Red. See Sudan IV.

Scharlach R. See Sudan IV.

Silver Gray. See nigrosin, water sol.

Solid Green. See malachite green.

Soluble Blue 3M or **2R.** See aniline blue, water sol.

Spirit Blue. (Syn. *Gentian blue. aniline blue, alcohol sol. night blue. Lyons blue. Paris blue.*) A basic dye of the triphenyl methane group (from which aniline blue, water soluble, is derived by sulphonation). It is a mixture of diphenyl and triphenyl rosanilin, and hence varies in shade according to the proportion of each present. It is seldom used in microscopic work; but has been employed in contrast to carmine in staining embryonic tissues. It brings out growing nerve fibers well.

Steel Gray. See nigrosin, water sol.

Sudan II. See oil red O.

Sudan III. (Syn. *Sudan G. Tony red. Scarlet G.* or *B. Fettponceau G. Oil red. Cerasin red.*). A weakly acid, fat-soluble dye of the azo group, which colors fat an orange color. It is the first of the oil-soluble dyes to be employed by histologists as a stain for fat in sections. It is still used, partly for staining sections and partly for staining fat in masses of tissue for gross examination; but to a large extent it is now replaced by Sudan IV.

Sudan IV. (Syn. *Scarlet red. Scharlach R. Oil red IV. Fettponceau. Ponceau 3B.*) A weakly acid, oil-soluble dye of the azo group, a dimethyl derivative of Sudan III. On account of the methyl substitution, it is of a scarlet rather than an orange color and accordingly stands out more vividly in a microscopic preparation. It is used as a fat stain in histology.

Sudan AX. See oil red O.

Sudan X. See oil red O.

Sudan G. See Sudan III.

Sudan Red. See Magdala red.

Sultan 4B. See benzopurpurin 4B.

Swiss Blue. See methylene blue.

Tetrachrome Stain (MacNeal). A mixture of methylene blue, methylene azure, methylene violet, and eosin, used for staining blood films. Recently MacNeal has specified azure A for methylene azure (see azure A). As these dyes are mixed in dry form, no reaction takes place between them. The mixture is dissolved in methyl alcohol, and no immediate reaction takes place. A compound dye is slowly formed, however, with the result that a troublesome precipitate may occur. Accordingly the best way to dissolve the stain is to allow the solution to stand for about twenty-four hours at approximately 50°C. so that the interaction between the dyes will take place as rapidly as possible; and then the precipitate can be removed by filtering. Such a solution keeps some time; but it is not as permanent as Wright's stain. When fairly fresh, it gives as good results as the latter; in fact the blood pictures obtained by the two stains are very similar. The chief advantage of the tetrachrome stain is that it can be prepared from the constituent dyes with a little more precision than Wright's stain.

Thionin. (Syn. *Lauth's violet.*) A basic dye of the thiazin series, differing from methylene blue and the azures in not being methylated. Thionin is no longer used as a textile dye; and until recently it was difficult to obtain the correct product when ordering. That difficulty no longer exists, however. It is a very valuable chromatin and mucin stain, especially prized for certain procedures because of its high degree of metachromasy. It is a useful vital stain. It is employed in staining frozen sections of fresh animal or human tissue, especially in the study of tumors. It is used for staining very young bacterial colonies in the Frost "little plate" technique for counting bacteria.

Thymol Blue. An acid dye of the sulphonephthalein series, employed as an indicator, changing from red to yellow through the H-ion range between 1.2 and 2.8, and from yellow to blue between 8.0 and 9.6. May be used as an indicator of reaction in vital staining.

Toluidin Blue O. (Syn. *Methylene blue O.*) A basic dye of the thiazin series, closely related to methylene blue, and even more like azure A. Theoretically it should be a good substitute for the latter; and in actual experience it proves possible to employ it instead of azure A in certain procedures called for by the latter. Further investigation may show that it can be substituted for the latter quite generally; and as it is a recognized textile dye, it will be much more readily available. It can be substituted for thionin in staining frozen sections of tissue. It has been definitely specified in certain staining procedures such as in the panchrome stain of Pappenheim (p. 247) and in the Albert stain for the diphtheria organism.

Toluylene Red. See neutral red.

Tony Red. See Sudan, III.

Tropeolin D. See methyl orange.

Tropeolin OOO No. 2. See orange II.

Trypan Blue. (Syn. *Chlorazol blue 3B. Benzo blue 3B. Dianil blue H3G. Congo blue 3B. Naphthamine blue 3BX. Benzamine blue 3B. Azidine blue 3B. Niagara blue 3B.*) An acid dye of the azo series, which has been occasionally employed in vital staining.

Trypan Red. An acid dye of the azo series, which has been used for vital staining.

Uranin. See fluorescein.

Vesuvin. See Bismarck brown Y.

Victoria Rubin. See amaranth.

Violet C, G or **7B.** See crystal violet.

Violet R, RR, or **4RN.** See Hoffman violet.

Vital Red. (Syn. *Acid Congo red. Brilliant Congo red.*) An acid dye of the azo series. It is quite an important vital stain.

Water Blue. See aniline blue, water sol.

Water Soluble Eosin. See eosin Y.

Wool Orange 2G. See orange G.

Wright Stain. The compound of methylene blue and eosin most commonly employed for staining blood smears. In preparing Wright's stain, the methylene blue is heated in flowing steam for an hour, by which treatment it is partly oxidized into methylene violet and the azures—i. e. "polychromed." As the dyes produced in this oxidation process are all basic, they are capable of entering into chemical combination with eosin. These compound dyes are insoluble in water and are therefore precipitated. For use, they are dissolved in methyl alcohol; and after the alcoholic solution has stood a short time on the blood film it is diluted with water or with a solution of buffer salts. In this mixture of alcoholic and aqueous solutions there are likely to be present the original dyes, eosin and methylene blue, also the oxidation products of the latter, and the eosinates of the various basic dyes in the polychrome methylene blue. As a result, a great variety of staining effects is produced. Wright stain may be produced in the laboratory from methylene blue and eosin, or it may be purchased ready made. The prepared Wright stain now obtainable on the market is very reliable, and is so inexpensive that its preparation by the biologist himself is not necessary, unless it is to be used in large quantities. (H. J. C.)

Aniline Stains: General Considerations

Aniline dyes may be treated very much as a class and general statements may be made which will therefore be applicable to all of them. They offer a great range of choice in both color and action and now constitute a very valuable resource in microtechnique.

(*a*) *Solvents.* Most of the anilines are soluble in both water and alcohol. If alcohol is used it may be in strengths of 30 per cent, 50 per cent, 70 per cent or 95 per cent. There are some general advantages in a solution made in 50 per cent alcohol, but on the other hand, especially for counterstaining, it is of advantage to use 95 per cent or even absolute alcohol. In addition to plain aqueous solutions there are those made in aniline water, which has the advantage of greater solubility and also of some mordanting action. Finally, it is often convenient to dissolve the aniline color in clove oil so that staining and clearing may be accomplished at the same time.

(*b*) *Strength of Solutions.* The formulas call for different percentages of aniline dyes, but for most purposes it is very convenient to employ saturated solutions. Since generally this approximates the 1 per cent to 2 per cent strength which is called for, it is perhaps the easiest manner of handling the dye.

(*c*) *Method of Application.* The anilines, like other dyes, are employed as both progressive and regressive stains, but, because of their solubility and the absence of mordants in most cases, they are almost perforce regressive stains. Differentiation may be accomplished by the use of the solvent which was employed in making up the dye, i. e., either water or alcohol. In case of refractory dyes where plain alcohol or water do not work rapidly enough, the addition of 0.1 per cent of hydrochloric acid will facilitate

extraction of the color. Where it is desired to introduce two colors into the preparation it is sometimes possible to discharge the first color from portions of the cell or tissue by the use of the second color. Commonly the basic nuclear stain is displaced by the plasma stain. Thus, if gentian violet is used as a chromatic stain it may be differentiated by the action of orange G.

Clearing is accomplished by the use of any of the ordinary reagents like xylol, but for many purposes where additional differentiation of a precise sort is required, a final step may be taken by the use of clove oil, which clears and differentiates at the same time.

(*d*) *Relation of Stain to the Fixative.* The fixative has, in many cases, an outstanding influence upon the operation of the dye. In general, aniline colors are most effective after fixatives containing chromic and osmic acid. The picro-formol-acetic mixtures are less effective in preparation for aniline dyes than are the osmic or chromic mixtures. In case it is desired to have the advantages of the picro-formol-acetic mixtures and at the same time utilize aniline colors, the end may be attained by adding a small quantity of Flemming's fluid to them. Sometimes it is even possible to mordant sections fixed in the P.F.A. mixtures by treating them with Flemming's fluid before staining.

(*e*) *Mordanting.* In general the aniline dyes do not require mordants, but for some purposes it is advantageous to attach the dye more strongly to the tissue than is possible by direct action and in this event a mordant, such as aniline water or a solution of potassium permanganate, is advantageous. The mordant is applied as in the cases of carmine and hematoxylin. If potassium permanganate is used, a 1 per cent aqueous solution, acting for five or ten minutes, is usually satisfactory. Iodine may also be used as a mordant. Instead of applying the mordant before the stain, it has been recommended that in the case of iodine it be allowed to operate after the stain is in the tissue.

2. Inorganic. Inorganic staining agents are mostly salts of gold and silver which under the action of light are precipitated as metals in the presence of certain substances, like intracellular cement, and within nerve and neuroglia cells. Such effects are commonly spoken of as impregnations and differ materially from the ordinary staining reaction, although they grade into it under some conditions. Since they are used to demonstrate tissue and cellular constituents they may be properly considered along with ordinary stains.

II. Composition of Staining Agents

1. Physical Composition. In their physical state staining agents may be either *simple* or *compound*, that is, they may consist of a single constituent or of more than one element. A consideration of this phase of the subject is of value only in the way of orientation.

2. Chemical Composition. In chemical constitution stains may be either acids, bases or salts, or they may be of complicated organic structure. In

general they do not exhibit any pronounced acid or basic properties. Sometimes the salts are designated as basic or acid dyes, depending upon whether the radical upon which their staining power depends occupies the position in a compound of a base or an acid. (For discussion of these topics in detail see the sections on the different dyes pp. 437, 460.)

B. NATURE OF STAINING COMBINATION

I. Physical Nature

After the stain has been dissolved in a medium there are certain physical characteristics with regard to it which have important bearings upon its action.

1. **Solvent Aqueous or Alcoholic.** The solvent, whether water or alcohol, while it does not alter the characteristic staining reaction of the dye, does have a material effect upon the rapidity and degree of its action. Accordingly a solvent should be chosen which comports most favorably with the particular staining reaction desired. In most cases an aqueous stain is preferable, but sometimes it is quite advantageous, especially in counterstaining, to make the application of the dye late in the process, just before mounting. In this case, of course, an alcoholic solution should be made, or even one in clove oil when it can be used for clearing.

2. **Concentration, Strong or Weak.** The concentration of the dye in the medium also influences the character of the stain. As a general rule, in progressive staining, a weak solution of the dye, allowed to act over a longer period, gives a more precise and selective effect than when a concentrated solution, acting more rapidly, is used. On the other hand, when it is difficult to secure a reaction between dye and tissue, use a concentrated solution. The solvent effect of the dehydrating agent or clearing agents then produces sufficient differentiation. In very resistant cases a solution of an aniline dye in aniline water is more effective than is a plain aqueous solution.

3. **Temperature, Low or High.** The temperature of the solution when staining takes place also influences the nature of the stain. Particularly in the case of blood smears, the temperature of the reaction is important (see Blood, p. 248). Commonly, however, the staining operation takes place best at room temperatures and unless there are particular reasons for doing otherwise, this should be the practice.

4. **Composition, Simple or Multiple.** Staining solutions in their compositions may be either simple or multiple, that is, they may consist of a solution of one dye, or there may be two or more of these. By such combinations it is possible to get contrasting colors combined with different portions of the tissue or cell at one operation. These special combinations will be considered particularly under different headings (see Blood, p. 244). Some of these combinations are more than mere mixtures, being in effect

chemical unions between a staining base and a staining acid of different colors. Polychromatic or metachromatic effects of beautiful clearness result from the use of such stains.

II. Chemical Nature

1. Acid, Basic or Neutral. The chemical composition of combinations, quite aside from the chemical reaction of the active dye, may be either acid, basic or neutral. Sometimes, as in the aceto-carmine mixture of Schneider, the acid is present in strong concentration and so acts as a fixative. Similarly, methyl green combined with acetic acid forms a strongly acid combination which fixes and stains simultaneously. Various combinations are rendered acid in order to secure greater vigor and precision in their effects. On the other hand, only a few staining combinations are basic. The most outstanding examples of such a stain are Beal's borax carmine and ammonia carmine. These alkaline stains were in the early days much used, but at present are only infrequently employed. Most stains are now made up in neutral media.

C. APPLICATION OF STAINS

I. Intra Vitam

Intra vitam stains are those which are applied to the tissue in its living condition. Such stains are called "vital" stains or "supravital" stains. For discussion of intra-vitam stains see chapter on fresh material (p. 82).

II. Post Mortem

Post-mortem application of stains, or their use on tissues after they have been killed and fixed, is the common practice; therefore most of the discussion in this chapter will be on this subject. The practical application of stains in this way is influenced by (a) the character of the material and (b) certain relations to the stains.

1. The Character of the Material to Be Stained. This may be indicated under the categories of whole organisms, whole organs, tissues, or cells.

a. Whole Organism, Entire Organ, Tissues and Cells. If the organism is small it is convenient, and in every way desirable, to stain it entire. This obviously has many advantages because the parts are all seen in their normal relations and, by the selection of proper staining agents, may be well differentiated from each other. In general, the best stains to use for such a purpose are the carmine mixtures, either alone or combined with picric acid. Sometimes it is desirable similarly to study single organs entire and they may be treated in the same manner. In the majority of cases, however, the differential staining reaction takes place upon tissues or cells, that is, the stains are classified as either histological or cytological. It is possible to differentiate well between tissues in a very striking and characteristic manner as is done, for instance, by the use of Mallory's connective

tissue stains, or the various neurological stains. Finally the parts of cells may be distinguished by specific staining reactions. Perhaps the most general and characteristic of such differential effects is that obtained by the use of nuclear stains which pick out these cellular elements by their specific staining reactions.

2. **State of Material to Be Stained.** *a. In Toto.* Staining in toto was formerly much practiced, but latterly has fallen into considerable disuse. This method, however, has certain advantages which, if carefully utilized, make it one of great convenience. It is perhaps most commonly used in embryological work where the whole organism is stained before it is sectioned. The value of this method depends largely upon the character of the staining agent and the choice here is rather strictly limited. Neither the aniline stains nor hematoxylin lend themselves well to such use. There remain, therefore, practically only the carmine combinations. Perhaps the best of these, for most purposes, is not a combination including carmine itself but rather the alum-cochineal stain. This gives a precise effect and also has the advantage of not overstaining. Properly controlled, a very exact differentiation, not only between tissues but also between cellular elements, can be secured (alum-cochineal).

b. Sections. Sections are usually selected as best for application of stains. If the *paraffin* method has been used, the sections are affixed to the glass slip before staining, whereas most *collodion* sections are stained before they are mounted. This is not obligatory, however, and if serial sections are prepared by the collodion method it is almost necessary to affix them to the glass before staining is attempted. For description of method of staining sections see Part 1.

c. Dissociated. Dissociated materials, as a rule, stain with difficulty because the dilution of the fixing agent required for securing the macerating effect results in an indifferent staining reaction. It is therefore usually desirable to use a vigorous stain like Delafield's hematoxylin, although by proper treatment picro-carmine may be made to render an acceptable result. After the fixative is washed out the stain is added to the fluid in which the cells are floating and allowed to act until the desired effect is produced. It is sometimes convenient to continue this treatment through all the successive steps involved in mounting in balsam. When this is done, a drop of balsam containing the cells, when placed under a cover glass, will give a complete preparation.

d. Stretched. Stretched material is essentially the same as sections except for the fact that there are no cut surfaces through which the stain can penetrate. The methods employed for sections can therefore be applied directly.

3. **Relation of Material to Stain.** The relation of the material to the stain may be influenced by the character of its action and by the time and degree of action.

a. In Action. In action stains may be substantive, adjective, or impregnation stains. *A substantive stain* is one which acts immediately and directly upon the tissue without the intervention of any other substance. *An adjective stain* is one in which the tissue is first treated with some agent which in turn attaches the stain to the tissue. *Impregnations* are deposits of sensitive metallic substances in tissues under the action of light.

b. In Time. Relation of material to stains in time of action. The character of staining depends upon the time of its action on the tissue. A selective action upon any one tissue or cell constituent may be obtained by watching the degree of stain. By observing sections under the microscope, therefore, the desired differentiation is secured directly.

Progressive Staining. Such a practice is known as progressive staining. For many purposes the precision thus reached is satisfactory, but it is obvious from the nature of the process that there must be a gradation of effect which would prevent the sharpest differentiation.

Regressive staining, therefore, is resorted to when the most extreme sharpness of differentiation is desired. By this method the whole tissue is completely stained and then, by removing the excess dye from the parts desired unstained, by careful control under the microscope, an extreme precision of differentiation may be attained. Such a method may be applied to all classes of stains. It is the one which is utilized in the classical method of Heidenhain in his iron alum-hematoxylin combination. It is almost obligatory in the case of aniline stains because, in the process of dehydration, and to some extent in that of clearing where essential oils are used, a differential solution of the stain is necessarily accomplished.

c. In Degree. In the degree of their action upon tissues, stains may be classified as general or selective.

A general stain is one which attacks all parts of the tissue with approximately equal vigor and thus produces no marked differentiation. Sometimes by regressive methods a general stain may be made to exhibit more selective action. Such general stains are more commonly found among the anilines than in natural stains. Much of selective action is due to previous treatment of the material and to method of applying the stain rather than to inherent differences in dyes.

Selective stains may differentiate between classes of tissues or between parts of cells. Certain stains attack only particular tissues, as orcein does elastic tissues, or Sudan III fat, etc.

Plasma and Nuclear. In cytological work there is a series of stains which have specific effects. Although we have no exact chemical tests, cytological stains generally fall under the headings of nuclear or cytoplasmic. Nuclear stains are primarily those which affect chromatin, although there are certain ones which, in a measure, have a differential action upon the so-called achromatic structures. The natural stains are primarily nuclear stains and this is true also of basic aniline dyes. Of stains differentiating

cytosomic structures there is correspondingly a whole series. Many of the aniline dyes may be utilized for differentiating the general cytoplasm as opposed to the nucleus. There are, however, certain more specific agents which are used for distinguishing mitochondria and the Golgi apparatus. This subject will be found discussed more in detail in the section dealing with these topics (p. 205). Certain aniline dyes are only slightly toxic and have specific selective action on constituents of living cells (p. 82).

D. FORMULAS

I. Aniline Dyes

Single aniline dyes are simple solutions in either water, alcohol or clove oil. For stronger action, aniline water (distilled water saturated with aniline oil) is used as the solvent. The strength of the solution, except for the vigor of the action, is not important, since differentiation always attends dehydration. There are, however, a number of combination stains which are of great value and which sometimes are of exact composition. Because of variations in materials to be stained and uncertainty in the chemical constitution of aniline dyes it is difficult to give exact directions regarding strength of solutions and relative time of action where more than one dye is used. The only practical plan when trying new dyes on new material is to test out the solutions and their operation until the desired result is obtained. There are a very large number of multiple aniline stains, only a few of which have general application. Among these are the following:

Flemming's Tri-color Stain. Although according to the dictum of Lee "never popular, this clumsy and uncertain process is now little used," this method is one of the most useful and popular of the combination aniline stains. It admirably supplements the Heidenhain iron hematoxylin method and has some advantages over it in the contrasting colors shown in prophases of mitosis and in the transparency of the metaphase chromosomes. As suggested elsewhere it should form a routine alternative to the use of the hematoxylin stain in cytological work. There are many modifications of the strengths of solutions and methods of application, but these are of little consequence since they represent adaptations to particular cases. With the proper choice of aniline dyes to begin with, the details of strength of solution and time of application can easily be adjusted to each case. Due regard should be had to the character of the illuminant when the safranin and gentian violet are chosen (p. 471). The following solutions will be found satisfactory in the majority of cases:

(1) Saturated alcoholic solution of safranin, 95 per cent..... 1 part
Aniline water..... 1 part

(2) Gentian violet, saturated aqueous solution

(3) Orange G, saturated aqueous solution

Sections fixed in either Flemming or Hermann, or after fixation in other fluids mordanted in osmic acid solution, are stained for two to twenty-four hours in the safranin.

They are then rinsed in water, and transferred to the gentian violet solution for an appropriate time, for example, thirty minutes to two hours. They are then removed from the staining jar and on them is dropped the orange G solution which is allowed to act thirty to sixty seconds. The object generally aimed at is to secure a stain which shows the chromosomes in metaphase stained clearly with the safranin, while the prophase chromatin takes the gentian violet. The purpose of the orange G is not so much to add a third color, but rather to differentiate between the red and the violet dyes.

Differentiation is accomplished by dropping on the slide 95 per cent alcohol until clouds of color cease to come from the sections. After this treatment, clove oil is dropped on the sections, and final differentiation accomplished by observation under the microscope. The action of the clove oil is slow, so that a very exact differentiation may be secured. Some workers prefer to differentiate the safranin before adding the gentian violet and there are various other modifications of the process, but the end-result sought in each case is to obtain in different portions of the cell accurate segregations of the two principal colors in the stain.

The action of this stain is so precise under proper manipulation that in one strand of chromatin the diffused portion will take the violet, while the condensed section will be stained with the safranin.

Wright's stain (Blood, p. 245).

Leischmann's stain (Blood, p. 244).

Romanowsky's stain (Blood, p. 244).

Van Gieson's stain (Section on Connective tissue, p. 289).

Mallory's aniline blue connective tissue stain (Section on Connective tissue, p. 308).

Mallory's acid fuchsin (Section on Connective tissue, p. 288).

Feulgen's reaction, p. 483).

II. Carmine and Cochineal

1. Carmine Stains, Alkaline and Neutral.

a. Ammonia-carmine. Ranvier introduced a stain prepared by dissolving carmine in water with a slight excess of ammonia. Upon evaporation a substance is produced which, when subsequently dissolved in water, makes an excellent stain.

*b. Picro-carmine.** In a saturated solution of picric acid dissolve carmine until no more is taken up. Evaporate to ⅕ of the original volume and filter off the precipitate which forms when the liquid cools. Evaporate the filtrate to dryness, which will give a crystalline powder somewhat the color of red ochre. For use prepare a 1 per cent solution of this.

c. Alcoholic Carmine Stains. Borax-carmine. In a 4 per cent solution of borax dissolve 2 per cent of carmine by boiling for half an hour. Allow to stand for two or three days and dilute with about an equal volume of 70 per cent alcohol and then filter. After staining, bring the material into 70 per cent alcohol acidulated with 5 drops of hydrochloric acid in each 100 c.c. of alcohol. When the material has become clear and transparent wash out the acid in alcohol. This is very valuable for staining in bulk.

2. Carmine Stains, Acid.

a. Alum-carmine.† In 5 per cent aqueous solution of ammonia alum boil for ten to twenty minutes 1 per cent of powdered carmine. Cool and filter. This is very easy to use since it will not overstain.

* Ranvier, Traité, p. 100.

† Grenacher, *Arch. f mikr. Anat.* 1879, xvi.

*b. Carmalum, Mayer's.** In a 5 per cent aqueous solution of ammonia alum dissolve ½ per cent of carminic acid, using heat if necessary. Filter and add 0.1 per cent of salicylic acid. This is very valuable for in toto staining. Wash out the excess of stain in distilled water which will leave the cytoplasm colored. If a purely nuclear stain is desired use an alum solution for washing.

c. Alum-carmine and Picric Acid.† Mix 10 volumes of alum carmine with 1 volume of saturated picric acid solution. With this a double stain may be secured.

d. Schneider's Aceto-carmine. Heat 45 per cent strength acetic acid to the boiling point and dissolve in it as much carmine as it will take up. Filter and preserve the filtrate. This is a very valuable stain of somewhat limited application. Fresh material may be fixed and stained at the same time but the specimen cannot well be preserved afterward. It stains the nucleus almost alone.

A modification of this stain has been used recently by Belling. His formula is as follows:

Mix equal volumes of glacial acetic acid and water. Add to this powdered carmine in excess. Boil, cool and filter. To the solution add a few drops of ferric hydrate dissolved in 50 per cent acetic acid. This acts as a mordant and may be varied in proportion to suit the requirements of different objects. The amount of iron affects the color of the stain. The greater the proportion of the iron, the darker and bluer the stain. This is particularly valuable in making rapid studies of chromosome numbers in smears. The preparation may be examined directly in the stain. Such preparations are fugitive but may be preserved for a considerable time by sealing the edges of the cover glasses with vaseline or some other substance.

3. Alum-cochineal.

Powdered cochineal	12 gm.
Potassium alum	12 gm.
Water	160 c.c.

Dissolve the alum in the water and boil the cochineal in the mixture for twenty minutes. Decant the clear supernatant liquid, add water to the cochineal, and boil again. Decant and add to the first liquid, filter and evaporate to 160 c.c. Add a crystal of thymol to prevent the growth of molds. This is largely used for staining in toto. For this purpose place the piece of tissue in the stain for twenty-four to forty-eight hours. Wash in water twenty to sixty minutes to remove the alum, but in order to avoid the extraction of the stain run up through the grades of alcohol to 70.

III. Hematoxylin and Hematein

a. Delafield's hematoxylin.‡ Prepare 400 c.c. of saturated solution of ammonia-alum. To this add 4 gm. of hematoxylin crystals dissolved in 25 c.c. of 95 per cent alcohol. Expose to the light and air for three or four days. Add 10 c.c. of glycerin and 100 c.c. of methyl alcohol and filter. By being exposed to the air it will slowly ripen, but this process may be rapidly completed by adding a small quantity of hydrogen peroxide. This solution keeps indefinitely. In the undiluted condition it is a very powerful and somewhat general stain, but in dilute solution it acts more slowly and specifically. This is one of the most common stains used in clinical studies and is a valuable laboratory reagent. Very often it is used with eosin as a counterstain, giving a strong contrast between the nucleus stained blue with hematoxylin and the cytoplasm pink with eosin.

* Mayer, *Mitth. Zool. Stat. Neapel.*, 1892, x.

† Legal, *Morph. Jahrb.* viii.

‡ Delafield, *Ztschr. f. wissensch. Mikr.*, 1885, ii.

*b. Mayer's hemalum.** Dissolve 1 gm. of hematoxylin in 1 liter of water. To the solution add 2 gm. of iodate of sodium and 50 gm. of alum. This is an improved formula of Mayer's original hemalum. It acts very much like Delafield's but the solution does not keep so well.

c. Mayer's acid-hemalum. This is a stain made by adding 2 per cent of glacial acetic acid to the hemalum stain.

d. Von Möllendorff's iron hematoxylin (p. 253).

e. Mallory's phosphotungstic acid hematoxylin (p. 288).

f. Mallory's phosphomolybdic acid hematoxylin (p. 290).

g. Mallory's ferric chloride hematoxylin. Mordant in 10 per cent ferric chloride for five minutes; rinse quickly and stain in 1 per cent hematoxylin, five minutes, and differentiate in 0.25 per cent ferric chloride (See Heidenhain's stain below).

h. Verhoeff's stain (p. 296).

i. Heidenhain's Iron Alum Hematoxylin.† This is used in two solutions: (1) a mordanting solution consisting of a 4 per cent aqueous solution of ferric alum (ammonioferric sulphate). In preparing this be careful to select crystals that are all a pure violet color and not oxidized. (2) A ½ per cent aqueous solution of hematoxylin—best prepared by first dissolving the hematoxylin crystals in a small quantity of 95 per cent alcohol. In use the sections are first placed in the mordanting solution for a period of time running from one-half hour to twenty-four hours. From this they are brought into water where the excess of the alum solution is removed. This is important, since if the alum is carried over into the hematoxylin solution it injuriously affects it. After washing, the sections are brought into the hematoxylin solution where they are left for a period of time equal to that of mordanting. At the end of the operation they will be entirely black throughout without any differentiation. It is then necessary to remove the excess stain, which is done by use of the mordanting solution. If this works too rapidly it may be diluted to 2 per cent strength. This operation must be watched carefully under the microscope and at its conclusion the sections are thoroughly washed in water (fifteen to sixty minutes). This is important. The character of the stain seems to be improved by dipping the sections into water at intervals during the process of differentiation. The easiest way to accomplish this is to examine the sections in the water rather than in the alum solution. This is perhaps the best cytological stain which we possess, since it is very precise and exact in its operation, and, because of the method of differentiation, is very flexible. The length of mordanting and staining has some effect upon the final result. Longer treatments give a greater range of action. The color is somewhat affected by the length of treatment also. In this respect, however, the most pronounced effect is due to the age of the solutions, particularly of hematoxylin, fresh stains being bright blue, while older stains range towards black and finally to a rusty green color.

j. Conklin's Modification of Delafield. A valuable stain for eggs, where the presence of yolk masks nuclear structures, because of its affinity for hematoxylin.

Take 10 c.c. of Delafield's hematoxylin and 40 c.c. of water, and add 10 drops of Kleinberg's picro-sulphuric acid mixture. Stain sections for five or ten minutes and wash in alcohol. The presence of the acids in the mixture prevents the yolk from staining and allows the hematoxylin to act upon the chromatin alone.

Hematein Stains

a. Mayer's mucihematein.

Hematein	0.2 gm.
Aluminum chloride	0.1 gm.
Glycerin	40 c.c.
Water	60 c.c.

* Mayer, *Ztschr. f. wissensch. Mikr.*, 1923, xx.

† Festschr. f. Kolliker, 1892, iii.

Dissolve the hematein in the glycerin and add the other substances. (For the use of mucihematein, see p. 298.)

b. Orcein is a dye obtained from certain lichens such as Lecanora parella. It is of a violet color and gives metachromatic effects—nuclei blue and cytoplasm pink.

Orcein	2 gm.
Glacial acetic acid	2 gm.
Distilled water	100 c.c.

Wash in water, dehydrate, clear and mount in cedar wood oil. (For the use of orcein in staining elastic tissue, see p. 297.)

CHAPTER XI

MISCELLANEOUS

C. E. McClung and C. H. Heuser

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A. APPARATUS

I. Handling Small Objects

Oftentimes it is desirable to handle one or many very small specimens which are easily lost in handling. One of the best means of overcoming this difficulty is to use round bottomed shell vials or even a specially shaped one employed in centrifuging. The objects are brought to the bottom of the container, either by gravity or by use of the centrifuge, and the supernatant fluids withdrawn and new ones added. This is a very satisfactory arrangement for carrying through quantities of small eggs and other such substances. Another device employed by Boveri was to enclose the objects in a small packet of frog skin which was held together by a pin which also served as a handle to manipulate the bag. It is even possible by this arrangement to section the preparations and the enclosing material at the same time. Other membranes such as amnions may be employed in the same manner. For the special purpose of handling numbers of small eggs Lefevre devised a special form of watch glass with a small trough in the bottom, of a size which would be satisfactory for forming the block of paraffin in which the eggs are sectioned. With this special watch glass the eggs are more easily brought into a small compass and it has also the advantage that the final step of infiltration and imbedding may be accomplished in the same container. If a single small object is to be handled it introduces greater difficulties and many devices have been brought forward for overcoming them. (For a description of these see Orientation methods, p. 477.)

II. Illuminants in Relation to Stain

By due consideration of the relation between the color of stain and that of the light employed, greatly improved results can often be obtained. In the discussion of aniline dyes reference has already been made to the

desirability of choosing the right contrasting shades of red and violet for the tricolor stain to be used with artificial illumination. If a standard source of illumination is always used it is possible by proper tests to select a series of colors which will work best under these conditions. However, in the event that this has not been done, improvements may often be effected by the action of color filters; those of the Wratten and Wainwright series are readily available and in their cheaper forms offer no difficulties in the way of expense. By the use of a green color screen in connection with a red dye a very strong contrast approaching a black and white result is obtainable. These devices are of value in visual observation, but when it comes to the use of photography for making permanent records they are almost indispensable. By the proper selection of color filters a considerable range of effects may be secured from the same preparation which makes its interpretation very much more complete than does a single photograph. For a full discussion of such optical aids reference should be made to works on photomicrography.

III. Filtering Solutions

With the great magnification employed in microscopical work the presence of foreign substances is not only displeasing to the eye, but also very inconvenient or even detrimental in observation of the optical images.

While it is difficult to avoid these intrusions entirely their presence can be greatly reduced by careful filtering of the solutions employed, at frequent intervals. It is a good practice immediately before using a solution to run it through filter paper. Unless this is done as a matter of routine, operators grow careless and may suddenly find that a fungus has been growing in one of the stains and is present in unpleasant profusion in the preparations.

IV. Cements

For sealing temporary mounts vaseline or paraffin is used. A very convenient way of applying the latter is to take one of the small candles used for cake decorations, light it and allow it to burn until the paraffin melts and then, with the specimen all ready for ringing, the light is blown out and the wick is used as a means of applying the melted paraffin. For permanent mounts it is often of advantage to surround the mounting medium with a cement, although this practice is less in use than was formerly the case, because the modern mounting media are not so subject to change as was Canada balsam. Of the substances used for ringing covers asphaltum is one of the oldest and best. Orange shellac colored with Bismarck brown also makes an excellent ring. Solutions of celluloid in acetone or amyl acetate or butyl alcohol, or some of the trade preparations like Duco afford satisfactory results.

B. MATERIALS

I. Mounting Media

1. Aqueous. Some preparations are best preserved in aqueous rather than in balsamic mounting media. The commonest of these are Farrant's medium and glycerin jelly.

Farrant's medium

Gum arabic	30 gm.
Glycerin	30 c.c.
Arsenous oxide	0.1 gm.
Water	30 c.c.

Keep in stoppered bottle with a lump of camphor.

Glycerin jelly

Soak a quantity of gelatin in cold water for two hours.
Warm in a water bath until melted.
Add glycerin 1½ parts.
Filter and add a few drops of carbolic acid.

In the use of these it is of course unnecessary as well as undesirable to dehydrate the material. It should be brought either directly into the mounting medium from water or through a mixture of water and glycerin in the case of glycerin jelly. It is very difficult to prepare mounts of this character since, for best results, the slide should have built upon it a ring of cement of sufficient height to hold the requisite amount of mounting medium. After the excess is carefully removed, leaving the glass slip entirely dry, the mount is sealed by ringing it with cement.

2. Balsamic. The balsams are used as mounting media and the common ones are Canada balsam and damar. Sometimes colophonium or resin is employed for this purpose. It is important that only the best quality of these balsams be selected, since if there are acids or other impurities present, the stains are injuriously affected. Because it has been very difficult to get Canada balsam of a satisfactory character in recent years, the use of gum damar has much increased. In preparing this mounting medium select only the clearest and purest tears and dissolve these in benzol to form a thin syrupy solution. This is carefully filtered in order to remove all suspended matter and evaporated to a proper consistency. If it is found in mounting specimens that the benzol is too rapid in its evaporation the original solution may be reduced to a very thick syrup and diluted with xylol or toluol. If the mounts are small, and rapid hardening is desired, the solution in benzol is a very desirable one, but for ordinary purposes the xylol mixture seems to be the best. Other media such as chloroform or turpentine may be used as solvents, but the ones previously mentioned seem to be most satisfactory.

In order to prevent the formation of acid in the balsam it is sometimes wise to add a small quantity of some carbonate like calcium carbonate to the bottle of balsam.

II. Reagents

1. **Acetone** has only limited application in microtechnique. It is an almost universal solvent and because of this fact might have very important applications. On the other hand, its solvent power makes it difficult to utilize. It may be employed as a dehydrating agent in place of alcohol. It may also be used as a fixative but so far has not been highly recommended for this purpose.

2. **Alcohol.** Ethyl or grain alcohol is one of the most used substances in microscopical technique. It serves as a fixative, a solvent and a dehydrating agent. It is in the latter capacity that it finds the greatest employment. Educational institutions may obtain 95 per cent alcohol through the Internal Revenue Department by making application according to the requirements of the Department. Individuals can purchase 95 per cent alcohol only when it has been denatured. In some cases the denaturant injuriously affects the alcohol for technical purposes, but if nothing is added except methyl alcohol it is entirely satisfactory, provided the strength has not thereby been reduced. For most purposes at least 95 per cent alcohol is necessary for proper dehydration, although in case aniline oil is employed for clearing, much lower grades may be used.

Certain grades of methyl alcohol are quite satisfactory for technical use, but if grain alcohol can be obtained it is the best reagent. Absolute alcohol may be secured by removing the remaining water in 95 per cent alcohol by distillation over quicklime. A convenient method of doing this is to shake the alcohol up with lime and allow it to stand for some time. Then a quantity of this alcohol, together with some fresh lime, is placed in a still and by heating with an electric hot plate the alcohol is slowly distilled off leaving the water combined with the lime. Absolute alcohol should be carefully protected from contact with air because it very quickly takes up moisture and becomes reduced in strength. Another method of increasing the strength of alcohol without distillation is to treat 95 per cent alcohol with exsiccated copper sulphate. This takes up water from the alcohol and it is practical to operate by having some of the copper salt at the bottom of a bottle from which the alcohol is poured off as desired. This is less desirable than distillation over lime, since some of the copper remains in solution.

Exsiccated or anhydrous copper sulphate is prepared by driving off the water of crystallization with heat. A convenient way of doing this is to place the crystals of copper sulphate in a crucible which is then heated over a blast flame until the blue color is entirely removed, leaving behind a white substance. This is powdered in a mortar and it is then shaken up with the alcohol.

For some purposes absolute alcohol is not required. The commercial grade of 95 or 96 per cent is sufficiently high to secure dehydration. This is

especially true if the intermediate step of mixture of equal parts of dehydrating and clearing agent is employed.

3. **Acid Alcohol.** Acid alcohol is used for cleaning glass slips and also for decolorizing after the use of certain stains. Either 70 or 95 per cent alcohol may be used, but commonly the former. Various strengths are employed. A 1 per cent solution is particularly useful in decolorizing. The action of this is rather rapid on sections so the process of decolorization should be watched under the microscope.

4. **Alums.** These substances are double salts containing a large amount of water of crystallization. They are used in microscopical technique as mordants and to form lakes with the staining agents. The commonest in use is the aluminum potassium sulphate. When not otherwise indicated this is the form to be employed in making up solutions of hematoxylin. The aluminum ammonium sulphate or ammonia alum is a cheaper substance and may without injurious results be used as a substitute for the potash alum. Ammono-ferric sulphate, or iron alum, is a salt in which the place of the aluminum is occupied by iron. The crystals of this alum, instead of being clear and colorless, like the potash and ammonia alums, are violet colored. On exposure to the air they oxidize and thereby become unavailable for use in the Heidenhain technique. It is therefore necessary to select, out of the mass of crystals, only those which are perfect in color. This salt is very deliquescent and should be kept from exposure to moisture. In very warm weather it may melt down in its water of crystallization.

5. **Amyl Nitrite.** This substance is used as a dilator of the peripheral capillaries and is valuable preceding injections where it is desired to obtain a complete injection of the small vessels. This effect may be obtained by adding amyl nitrite to ether at the time of anesthetization.

6. **Benzol** (C_6H_6) is the lightest of the benzene ring series. It is used as a solvent for balsam and paraffin and may therefore be employed as a clearing agent. Because of its rather high volatility, however, it is not as frequently used for this purpose as is xylol.

7. **Formalin,** otherwise known as formol or formolose, is a 40 per cent aqueous solution of formaldehyde. It is used in various strengths commonly stated in terms of the aqueous solution. For fixation a 10 per cent aqueous solution is commonly used which is prepared by adding 1 part of formalin to 9 parts of water.

III. Preparation of Reagents

1. **Mayer's albumen** is a mixture of equal parts of fresh egg albumen and glycerin to which has been added some preservative like sodium salicylate or thymol. The proportions recommended by Mayer are 50 c.c. albumen, 50 c.c. glycerin, 1 gm. sodium salicylate. The original directions require the filtration of this mixture, but this is exceedingly difficult and time-consuming. It may be easily avoided by a simple expedient. Place the fluid in a tall

cylinder and shake vigorously until a great many air bubbles are included. These will rise to the surface and carry with them the fragments of membrane which it is desired to remove. This upper layer can thus be taken off, leaving behind a clear mixture quite as good as that obtained by filtration.

2. **Paraffin** of various melting points may be purchased, and, as was indicated in the description of the paraffin method (p. 6), the one utilized should be chosen with careful consideration of the density of the object and the temperature of the room in which the sections are cut. New paraffin is apt to crystallize more readily than that which has been used, and it is the practice of some technicians to melt the fresh paraffin and keep it in this condition for a considerable period of time so that it may become of a more waxlike consistency. It is possible to improve the consistency by the addition of castor oil or India rubber in small quantities. The paraffin which is trimmed from the blocks around specimens is returned to the jar and remelted. After considerable use paraffin becomes contaminated with foreign materials, in part from the specimens, and in part from the air, and it is necessary to filter these out. The best way to accomplish this is to melt the paraffin and pour it into a paper funnel which has had the apex cut off and in which a wad of absorbent cotton has been placed. If this is placed upon a heated radiator the paraffin will remain melted and in passing through the cotton will leave behind the foreign substances. It is very necessary to avoid the presence of hard gritty material in the paraffin which would injure the knife edge.

It is desirable after the paraffin has been melted and filtered to cool it rapidly by immersing the vessel in cold water. If volatile products are present these will be forced towards the center of the mass which will remain spongy. This portion may be cut out before remelting and thus improve the consistency of the paraffin.

Rubber Paraffin

Johnson's asphalt formula.*

Crude India rubber, cut small	1 part
Paraffin, melted and tinged an amber color with asphalt	99 parts

Heat to 100°C., twenty-four to forty-eight hours.
Remove supernatant fluid and cool. Use as ordinary paraffin.

3. **Preserving Fluids.** Tissues may be kept indefinitely in 70 per cent alcohol and this is a standard medium for the purpose. Several years' immersion in this fluid seems to have no bad effect upon delicate cytological details. If the picro-formol-acetic mixtures have been employed for fixation a gradual extraction of the picric acid will occur, which colors the alcohol but which apparently has no harmful effect. Solutions of formalin of 4 per cent also may be employed for preserving purposes. In general this seems to have no marked injurious effect.

* Johnson. *J. Applied Micro.*, vi, 2662.

C. METHODS

I. Decalcification

This is a process of removing the calcareous substances from such tissues as bone and teeth (bone and teeth, pp. 259, 269).

II. Orientation Methods

In many cases it is necessary to define the plane of future sectioning accurately before inclusion of the specimen in the imbedding medium. It is very difficult after imbedding to establish a plane of sectioning with regard to the particular elements of structure. Therefore, at the time of imbedding the material is so arranged that by placing certain guides in proper relation to the knife, the desired plane can be realized. As was indicated in the description of the paraffin method, this may be accomplished by placing the tissue upon a piece of paper to which it adheres and so arranging it that the paper is parallel to the desired section plane. In other cases threads containing lamp black are laid in the paraffin and after hardening these are drawn through, leaving marks indicating the plane of section desired.

Very small objects cause unusual difficulties in orientation and special means are necessary. One of these is described by Heuser as follows:

A binocular microscope is mounted on a long swinging arm, so that it can be moved over the dishes which hold the specimens. The latter are thus always kept in view while they are being changed from one fluid to another. When they have been carried up to paraffin they are handled with a long slender glass pipette which is wrapped with coils of small resistance wire and heated with an electric current of proper voltage. A small rheostat controls the strength of the current so that the temperature is maintained just at the melting point of the paraffin. The infiltrating dishes are kept at a uniform heat in a shallow box, open in front for manipulating the instruments and closed on the remaining sides with thin wood boards. An automatic electric hot plate provides a bottom for the box. The top is made of glass; above it is mounted a 100 watt electric lamp which supplies some of the heat for liquefying the paraffin and also gives light.

After the object has been thoroughly infiltrated it is imbedded beside a small stained bit of tissue, for example, a slender column cut from an embryonic liver. These orienting pieces of tissue are prepared in advance and imbedded. Then when one is needed the paraffin above it is melted with an electric needle, the working point of which is made of copper, and the specimen deposited at the lower end of the tissue guide. The specimen is finally arranged as desired in relation to the long axis of the orienting guide block. The specimen cannot be conveniently moved about with the same electric needle that is used to melt the paraffin since eddy currents are set up in the fluid, which are apt to carry the specimen far from the desired position. There is the possibility, too, of injuring the specimen by touching it with the hot instrument, although the temperature of it need not be much above the melting point of the paraffin. The specimen rests upon the solidified paraffin which obviates any danger of superheating. A very useful instrument is one made by soldering to the copper point of the electric needle a second point of iron or German silver wire so that the 2 points stand apart at an angle of about 90°. Either the hot cop per point or the warm iron point can be used as occasion demands by revolving the tool, both the tool and the specimen being under constant observation. With these devices

specimens as small as mammalian eggs can be placed in any desired position. Very small objects cannot be seen in the cold paraffin but since its position in relation to the guide block is known it is a simple matter to make the proper adjustments on the microtome. When the sections are cut those including the guide are discarded and only the ones carrying the specimen are mounted so that the slide is unincumbered with any foreign material.

Fry* describes methods of handling small objects in the various steps of fixation, washing, etc. For orientation he recommends imbedding in small water color dishes under a dissecting microscope, in such a manner that the bottom of the vessel indicates the plane of sectioning. This paper contains a list of references for other methods of treating minute objects.

III. Bleaching Methods

Specimens containing a natural pigment or darkened by the action of osmic acid may be bleached, either entire or after sectioning, in hydrogen peroxide, chlorine mixtures, or sulphurous acid.

1. Hydrogen Peroxide Method.

Hydrogen peroxide	3 c.c.
Water	97 c.c.

Allow the solution to act until desired effect is secured. After a time maceration begins and the material should be removed to alcohol.

2. Mayer's Chlorine Method.

To a few crystals of potassium chlorate in a bottle or test tube add 2 or 3 drops of hydrochloric acid. When chlorine fumes are evolved add 5 to 10 c.c. of 70 per cent alcohol. The specimens are transferred from a similar grade of alcohol to the chlorine mixture and left until bleached. Depending upon their size and density this period may vary from a few minutes to a day or two.

3. Sulphurous Acid Method.

To a small quantity of sodium sulphate dissolved in water add 2 to 4 drops of hydrochloric acid. Sulphurous acid is evolved and goes into solution.

IV. Huber's Water-on-the-knife Method for Cutting Paraffin Sections

A method has been devised by Huber for cutting paraffin sections, using a sliding microtome, with a knife wet with water, just as celloidin sections are cut with a knife wet with alcohol. Using this method it is possible to cut and mount serial sections of embryos or blocks of tissue with greater precision than is possible with the usual dry knife method. Moreover, much thinner sections can be cut, with the Huber technique. Some details, like those described here, have been added to the method as demonstrated by Huber, but the essential points are as they have been developed by him.

* Fry, H. J. *Anat. Record*, 1927, xxxiv, 245.

The knife is sharpened by honing first on a yellow Belgian stone, if necessary using water, and finishing on a blue Belgian stone. A hone which has been used with oil should be avoided, since the slightest trace of oil on the knife will prevent the water from extending down to the cutting edge of the knife. For the same reason the knife is not stropped on leather. It is essential that the water does not draw away but is kept in contact with the cutting edge of the knife, otherwise the sections, as cut, will not float upon the water. If the water does not flow down to its edge the knife is cleaned with absolute alcohol and a large camel's hair brush. The alcohol is washed off with water and finally dilute albumen solution is applied. A short segment of rubber tubing slipped over the end of the handle of a brush is a very useful instrument for stroking the edges of the knife, which is thus kept clean and wet. When the knife is locked in position, on the microtome, as much fluid is placed on it as will remain without running off. The lower surface is kept dry. When the knife is drawn across the paraffin block the section will be cut smoothly and accurately but it will roll up unless the corner first touching the knife is held down by the point of a small camel's hair brush. The brush is wet with water and gently stroked over filter paper to bring the hairs to a point, or the brush can be moistened and pointed with the tongue and lips; the latter method is very efficient but has the disadvantage that a few squamous epithelial cells are apt to be carried to the slide. The moist tip of the brush is then slightly pressed against the corner of the block and held there just for a fraction of a second until about the first millimeter of the section is cut, when it is pulled away and the section will then float upon the water. Then with a large camel's hair brush dipped in dilute albumen solution, so that it holds the maximum amount of fluid, the section is lifted up with a rolling motion of the brush and transferred to its proper position on the slip. The latter is best held on a wire frame made to fit in a large shallow Stender dish. The slip should be held at an angle of about 30° and a dilute solution of albumen added, to submerge the slide up to the band for the first row of sections. When these sections have been arranged, enough fluid is removed from the dish to expose a band for the second row and so on until the slip is filled with sections. The level of the fluid in the dish can be easily controlled by placing a second chamber on a stand, provided with a rack and pinion and connecting the two dishes with a flexible syphon. The albumen solution is prepared by adding about 9 drops of Mayer's egg albumen to 30 c.c. distilled water. The slip is wet with the solution and rubbed with the tubing mounted on the brush handle until the fluid clings to all parts and it is then put in position on the wire frame. As the sections are smooth when cut they require no spreading as ordinary paraffin sections do. The slide is removed from the frame, and the fluid drained off and placed on a warm plate to dry. (C. H. Heuser)

D. GENERAL

I. Alligation

1. Preparation of Elements in Mixtures. A convenient method of approximate accuracy for making mixtures of different substances is the following: Write the strength of the solution desired. Beneath this draw a line and at the two ends write the strengths of the solutions to be used. Subtract the smaller figure from the larger in each case and place the subtrahends at opposite ends of the line in reverse position, according to the following diagram:

	70
95	0
70	25

The results thus obtained will give the proportions of the 2 substances. Thus, for example, if it is desired to make the grade of 70 per cent alcohol from 95 per cent alcohol and water (represented by zero), subtracting zero from 70 gives 70. This is placed beneath the 95, the strength of the alcohol. Likewise 70 is subtracted from 95, giving 25, which is placed beneath zero. It is thus indicated that 25 parts of water and 70 parts of alcohol are required to make a 70 per cent solution.

II. Thickness of Sections

The thickness of sections employed depends largely upon the purpose in view. If it is intended to study the relations of elements to each other in a tissue such as the course of the blood vessels or nerve fibers, sections of considerable thickness are desirable, often up to 50 or 100 μ. For most histological and cytological purposes sections cut from 7 to 10 μ are satisfactory. For the finest cytological details sections as thin as 1 or 2 μ are often helpful. For the best staining results in general, sections should be so thin as to insure cutting through the surface of cells or even of their nuclei. Otherwise it is difficult to secure accurate detailed differentiation.

III. Handling Cover Glass Preparations

Many preparations are best made by attaching the material to cover glasses, either in the form of smears or as sections. These delicate pieces of glass are more difficult to handle than thicker slips, but there are devices which make it quite feasible to prepare material in this manner. If it is desired to study both sides of the preparation this may be accomplished by placing it between 2 covers. The method of mounting on cover glasses deserves a wider use than it now has. If only a few covers are to be handled at one time they may conveniently be passed through the different reagents

by the use of special forceps designed for this purpose. These are so formed that they rest upon the table conveniently while the cover glasses are immersed in the reagents. In the absence of such special devices the covers may be laid upon small corks and handled in this position, the solutions being placed upon the upturned specimens and removed therefrom by pipettes. In this case care should be exercised to avoid bringing the objects in contact with the edge of the cover which would permit the solutions to be drawn off. Where large numbers of covers are handled at once there are special racks for holding the covers which may be immersed in the various fluids used. One of these designed by Metcalf is constructed of glass and is a very convenient device.

IV. Preservation of Specimens

If material is to be kept for a considerable length of time there are two general alternatives. It may be run up into 70 per cent alcohol where it, in general, will remain indefinitely without change. In this case there is a necessity of having some means of handling the fluid and this is sometimes a disadvantage. It may be avoided by continuing the operation of infiltration with paraffin if this is the method to be employed for sectioning and in this medium also the material will remain indefinitely without change. The advantages of this arrangement are that the specimens are easily preserved and are in a condition to be immediately sectioned. If it is intended to preserve the material in 70 per cent alcohol, the best method is to place the specimens in small shell vials, stoppered with cotton, and filled with 70 per cent alcohol. These vials are then immersed in some convenient vessel and the whole covered with 70 per cent alcohol. A very convenient container is afforded by some forms of fruit jars which have lids that are easily sealed tight upon cushions of rubber. The use of corks should be avoided since the alcohol extracts tannin and in some cases injuriously affects the specimens. It is sometimes more convenient to use large shell vials in which a number of specimens are placed with layers of absorbent cotton between them. In this case each specimen of course should have its designating number attached in some manner which will avoid its displacement. If a large number of small vials are kept in one container a very convenient way of placing them for ready access is to stand them on end in rows, or in a spiral, proceeding from the center outward. Thin strips of paper between the rows keep them apart. The numbers of the first and last specimens of the series should be placed upon the outside of the jar. (See Botanical Methods, Chap. IV.)

V. Tinting Light Colored Specimens for Identification

Objects which have been fixed in Carnoy (p. 422), or sublimate mixtures, are often almost exactly the color of paraffin and are difficult to orient and handle in this case. A very convenient method for avoiding

this difficulty is to stain the material lightly and superficially with some bright colored dye like eosin. In case this is subsequently undesirable it may be removed from the tissue by treatment with acid alcohol.

VI. Air Bubbles

1. Air Bubbles during Section Spreading. If sections are allowed to remain too long beneath the lamp the air dissolved in the water will accumulate as small bubbles and will force up the sections above them abruptly and thus disturb the level of section. These bubbles may be avoided by spreading the ribbon more rapidly.

2. Air Bubbles in Damar. If the cover glass is properly placed over the sections the damar will be spread uniformly but if it is dropped or lowered unevenly air bubbles will be included. By pressing lightly on the cover glass these may be removed without injury to the section if there is plenty of damar present. However, if the amount of medium is slight and not sufficient to fill the space beneath the cover, large air spaces will be produced. These, of course, cannot be removed by pressure, but sometimes may be replaced by adding damar on the opposite side of the cover, forcing the air out. In the event that these spaces are very large it is safer to remove the cover by placing the slide in xylol and making a fresh effort.

3. Air bubbles in tissues arise in various ways. Exposure to the air in passing the material from one solution to another is often responsible for this difficulty, which can easily be avoided by gradual transfer of the specimen from one fluid to the other by substitution. Air bubbles also arise in the process of decalcification where they are, of course, unavoidable. Removal may sometimes be accomplished by placing the material in the fluid underneath negative air pressure. If they are situated deep in the tissue, however, this method may not work. If too much injury is not done the specimen, an opening made by a needle or fine scalpel may make it possible to remove the air.

VII. Adipose Tissue

A good demonstration of this tissue is afforded by the following process:

Stretch a piece of mesentery containing fat upon rubber rings (see Stretching method, pt. 1). Fix in 5 per cent formalin, rinse in water, stain in a saturated solution of Sudan III and 70 per cent alcohol, five or ten minutes. Differentiate in 70 per cent alcohol until only the fat cells are colored. Wash in water. Counterstain in dilute Delafield for five minutes. Wash in water. Mount in Farrant's medium (p. 473).

VIII. Artifacts

As has been intimated elsewhere, it is a question in the minds of some whether all of the figures produced in protoplasm by the use of fixatives are not of the nature of artifacts. In the opinion of those with most experience, however, it seems perfectly evident that the consistency and definite-

ness of the figures in the best preparations indicate the existence of structures very much of the same character as are present in living material. On the other hand, there are certainly some results following on the application of reagents to protoplasm which are definitely of an artificial character. Some of these have been mistakenly described in the literature as natural structures. Certain crystalline forms resulting from the use of corrosive sublimate closely resemble natural fibrous conditions in the tissues. Also the use of too much Mayer's albumen in mounting sections results in certain coagulation products within open spaces in the tissue which might be mistaken for natural structures. It is important to check up on any unfamiliar or unusual conditions in the sections to see if they may possibly be produced by the technique.

IX. Feulgen's Stain

It is a serious hindrance to an analysis of cellular constituents that we have no specific chemical tests for them. Thus, while the substance we call chromatin has affinities for certain dyes, none of them can be used as a definite test for it. Recently Feulgen* has presented a method which he considers capable of indicating chromatin by a specific reaction. It is carried out as follows:

1. *Fixation.*

Corrosive sublimate 6 per cent aq. sol.	98 parts
Glacial acetic acid	2 parts

Apparently the P.F.A. mixtures will also serve.

2. *Hydrolysis.*

Sections are exposed for two minutes to the action of cold dilute hydrochloric acid, followed by digestion for four to fifteen minutes in the same strength of acid at a temperature of 60°C.

Hydrochloric acid s.g. 1.19	82.5 c.c.
Distilled water	1000.0 c.c.

3. Rinse in cold dilute hydrochloric acid.
4. Rinse in distilled water.
5. Stain in fuchsin-sulphurous acid ninety seconds.

Basic fuchsin	1 gm.
Distilled water	200 c.c.

Boil, cool to 50°C., filter, add 20 c.c. of the dilute hydrochloric acid. When further cooled to 25°C., add 1 gm. of anhydrous sodium sulphite. When the solution is decolorized it is ready for use and should be kept in the dark.

6. Pass through 3 baths of dilute sulphurous acid.

Distilled water	200 c.c.
10 per cent aq. sol. of sodium sulphite	10 c.c.
Dilute hydrochloric acid	10 c.c.

* Feulgen, R. and Rosenbeck. *Ztschr. f. Physiol. Chem.*, 1924, cxxxv.
Feulgen-Brauns, F. *Arch. f. d. ges. Physiol.*, 1924, cciii, 415.

7. Rinse in distilled water.
8. Counterstain in light green.
9. Mount in damar.

The test indicates nucleic acid (Thymus-nucleic acid) by the presence of an aldehyde group, liberated in its hydrolysis with the dilute hydrochloric acid. Orthopteran spermatocytes treated in this way present essentially the same picture as that following the use of hematoxylin.

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